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Characterization of [beta]-methylcrotonyl-CoA carboxylase of tomato: a newly identified biotin enzyme in plants

Xun Wang
Iowa State University

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tomato, a newly identified biotin enzyme in plants**

Wang, Xun, Ph.D.

Iowa State University, 1993

U·M·I

300 N. Zeeb Rd.
Ann Arbor, MI 48106

Characterization of β -methylcrotonyl-CoA carboxylase of tomato,
a newly identified biotin enzyme in plants

by

Xun Wang

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirement for the Degree of
DOCTOR OF PHILOSOPHY

Department: Biochemistry and Biophysics
Major: Biochemistry

Approved: / /

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major\Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1993

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ABBREVIATIONS

ACCase	acetyl-CoA carboxylase
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
BCCP	biotin carboxy carrier protein
BSA	bovine serum albumin
bp	base pair
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E-64	<i>L-trans</i> -epoxysuccinyl leucylamide(4-guanidino)- butane
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
GCase	geranyl-CoA carboxylase
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
IPTG	isopropyl- β -D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
Leu	leucine
MCCase	β -methylcrotonyl-CoA carboxylase
MES	2-[N-morpholino]-ethanesulfonic acid

MOPS	3-[N-morpholino]-propanesulfonic acid
NBRF	National Biomedical Research Foundation
OAA	oxaloacetate
OADCase	oxaloacetate decarboxylase
PAGE	polyacrylamide gel electrophoresis
PCase	pyruvate carboxylase ⁺
PEG	polyethylene glycol
PMSF	phenylmethanesulfonyl fluoride
PPCase	propionyl-CoA carboxylase
RNAase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
TCase	(methylmalonyl-CoA:pyruvate) transcarboxylase
TEMED	N,N,N',N'-tetramethylethylene diamine
Tris-HCl	tris[hydroxymethyl]aminomethane hydrochloride
UCase	urea carboxylase
UV	ultraviolet light
x-gal	5-bromo-4-chloro-3-indoyl- β -D-galactoside

GENERAL INTRODUCTION

Overview

Biotin was originally discovered as the "protective factor" (protective factor X) from "egg white injury", which is a disease caused in human and experimental animals by feeding large amounts of uncooked egg white (Boas, 1927). The "egg white injury" is due to the existence of a glycoprotein, avidin, in egg white. Avidin binds biotin with extraordinary high affinity. The dissociation constant for biotin from avidin is approximately 10^{-15} M (Kögl and Tönnis, 1936). The biological function of avidin in egg white is unclear, but has been speculated to be for the protection of eggs from pathogens (Green, 1975). That nature chose avidin-biotin system as a defense mechanism suggests that biotin is important for life. Genetic studies have shown that mutations affecting biotin synthesis and utilization are lethal in bacteria (Eisenberg, 1987), yeast (Mishina et al., 1980), human (Wolf and Heard, 1989), and plants (Shellhammer and Meinke, 1990). These studies confirm the essential biological function of biotin. Due to its importance in biology, the structure, biosynthesis, mechanism of reactions and metabolic functions of biotin-containing enzymes have been investigated vigorously in animal and microbial systems in the last 50 years. Biotin has been found to be of central importance in lipogenesis, gluconeogenesis and the catabolism of branched-chain amino acids in animals and bacteria (Moss and Lane, 1971; Wood and

Kumar, 1985; Dakshinamurti and Bhagavan, 1985; Knoles, 1989; Mildvan et al., 1991). By comparison, the biochemistry of biotin and of biotin enzymes in plants have not been well studied. To fill this gap in our knowledge of biotin enzymes the study presented here has focused on the molecular cloning of cDNAs and genes coding for β -methylcrotonyl-CoA carboxylase (MCCase) from tomato and characterization of the structure and regulation of this newly identified plant biotin enzyme.

The Biotin Prosthetic Group

Biotin is also called vitamin H. It was first isolated from egg yolk in 1936 by Kögl and Tönnis (Kögl and Tönnis, 1936). Its structure was determined in 1942 (Du Vigaud et al., 1942) and was later verified by X-ray crystallography (Traub, 1956). As shown in Fig. 1, biotin is a bicyclic compound with fused ureido (imidazolidone) and thiophene, and an aliphatic carboxylic acid side chain. The function of the biotin prosthetic group in biotin enzymes is to act as an intermediate-carrier of a carboxyl group. The carboxylation site on biotin is at the N-1 position (Lynen et al., 1961). Biotin is covalently attached to enzymes through an amide linkage between the carboxyl group of the side chain of biotin and the ϵ -amino group of a lysine residue, forming biocytin (biotinyl-lysine). The maximum distance from the α -carbon of lysine to C-2 of the tetrahydrothiophene ring is 14 Å (Gregolin, 1968). This 14 Å arm could give the bicyclic ring sufficient freedom of translocation in the enzyme. This flexibility of movement is important

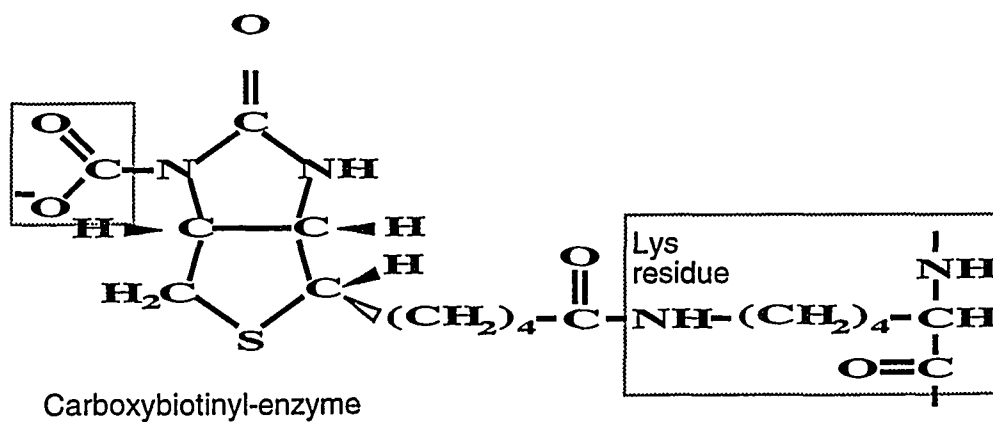
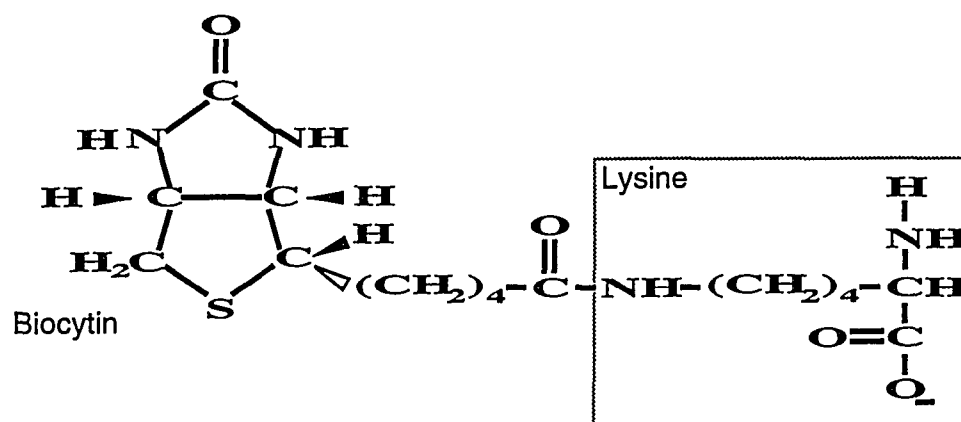
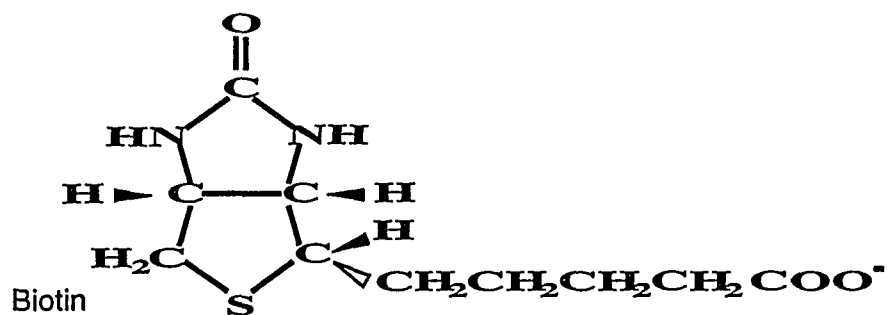


Figure 1. Biotin, biocytin and carboxybiotin-enzyme.
Biotin, Mr = 244.3; biocytin, Mr = 372.5.

for biotin enzyme action since biotin probably moves between two active sites during the catalytic reaction (Knowles, 1989).

Biotin Dependent Enzymes

The function of biotin in enzymology is to transfer CO_2 between substrate in a number of carboxylation, decarboxylation and transcarboxylation reactions. Thus biotin enzymes are accordingly classified into three groups: carboxylases, decarboxylases and transcarboxylases (Moss et al., 1971). To date up to nine biotin enzymes have been identified. In the carboxylase category, these are ACCase, PCCase, MCCase, PCase, GCase and UCase. In the decarboxylase category, they are OADCcase and methylmalonyl-CoA DCcase. The transcarboxylase category has only one member which is TCcase. The reactions catalyzed by these enzymes are involved in diverse metabolic processes. These biochemical pathways are fundamental to life, and include lipogenesis, gluconeogenesis, and the catabolism of branched-chain amino acids.

Although each enzyme has distinct metabolic function(s), in every instance the catalytic reaction mechanism can be explained by the role of biotin in these reactions. The role of biotin is to act as an intermediate-carrier of carboxyl group that is being transferred from one substrate to another (Moss and Lane, 1971).

The overall reactions catalyzed by biotin enzymes are carried out in two steps. The first step involves the carboxylation of biotin on the

enzyme. In the second step, carboxyl group is transferred from carboxybiotinyl enzyme intermediate to an appropriate substrate which serves as an acceptor (Knowles, 1989).

Structures of biotin-dependent enzymes

The two substrate binding sites and the biotin prosthetic group are reside on different domains or subunits of biotin enzymes. For example, ACCase from *E.coli* has three functional components:

1. Biotin carboxy-carrier protein (BCCP), on which the biotin prosthetic group is covalently bound to a lysine residue.
2. Biotin carboxylase, which catalyzes the ATP-dependent carboxylation of the BCCP-bound biotin in the first half-reaction.
3. Carboxyltransferase, which transfers the carboxyl group from the carboxylated biotin to acetyl-CoA to form malonyl-CoA in the second half-reaction.

All the three components are required for the overall reaction (Alberts et al., 1968; 1969; 1971; Guchhait et al., 1971). These functions are also separated onto three different polypeptides in the case of TCase (Wood et al., 1963; Ahmad et al., 1970).

The ACCases from *Streptomyces erythreus* (Hunaiti and Kolattukudy, 1982) and *Turbatrix aceti* (Meyer et al., 1978), however, have the three functions separate on two different subunits. The biotin-containing subunit has both the BCCP and biotin carboxylase functions, and the carboxyltransferase function is on a separate subunit. Such an

organization is also found in PCCases from animals (Halenz et al., 1962), MCCases from animals (Lau et al., 1979) and bacteria (Schiele et al., 1975; Fall and Hector, 1977).

In contrast, the ACCase from animals, yeast and plants (Takai et al., 1987; Lopez-Casillas et al., 1988) and PCase from animals and yeast (Lim et al., 1988) contain all three functions on a single polypeptide.

Based on these structures of biotin enzymes, Lynen hypothesized a gene-fusion mechanism by which the organization of biotin enzymes arose in evolution (Lynen, 1979). The hypothesis indicates that there may be two evolutionary steps for biotin-containing enzymes. The first is the fusion of the BCCP function with the biotin carboxylase function. The second is the fusion of the carboxyltransferase function with the fused BCCP-biotin carboxylase functions.

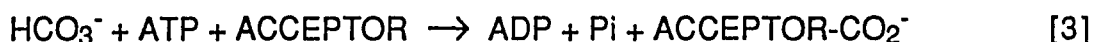
Support for this hypothesis comes from the sequence similarity that biotin enzymes exhibit. One sequence domain that is shared among biotin enzymes occurs at the amino acid sequence flanking the biotinylated lysine residue that is biotinylated (Fig. 2). The (Ala/Val)-Met-Lys-Met sequence is apparently absolutely conserved. However, this sequence is not the only structural information that defines the biotinylation site, since mutations away from this sequence motif affects biotinylation of this lysine residue (Samols et al., 1988).

pTBP	V	L	E	A	M	K	M	E	H	V	V
H-PC	V	L	S	A	M	K	M	E	T	V	V
S-PC	V	L	S	A	M	K	M	E	T	V	V
T-PC	V	L	S	A	M	K	M	E	T	V	V
A-PC	V	L	S	A	M	K	M	E	T	V	V
Y-PC	V	L	S	A	M	K	M	E	M	I	I
TC	V	L	E	A	M	K	M	E	T	E	I
<i>K. p.</i> -OADC	I	L	E	A	M	K	M	E	T	E	I
<i>S. t.</i> -OADC	I	L	E	A	M	K	M	E	T	E	I
H-PCC	V	I	E	A	M	K	M	Q	N	S	M
R-PCC	V	I	E	A	M	K	M	Q	N	S	M
<i>E. c.</i> -BCCP	I	V	E	A	M	K	M	M	N	Q	I
Ch-ACC	E	I	E	V	M	K	M	V	M	T	L
R-ACC	E	I	E	V	M	K	M	V	M	T	L
Y-ACC	E	I	E	V	M	K	M	V	M	P	L

Figure 2. Comparison of the amino acid sequences of biotin enzymes in their biotinylation sites. pTBP, tomato MCCase cDNA clone (see section I); H-PC, human PCCase; S-PC, sheep PCCase; T-PC, turkey PCCase; A-PC, avian PCCase; Y-PC, yeast PCCase; TC, *P. shermanii* transcarboxylase; *K.p.*-OADC, *Klebsiella* oxaloacetate decarboxylase; *S.t.*-OADC, *Salmonella* oxaloacetate decarboxylase; H-PCC, human PCCCase; R-PCC, rat PCCCase; *E.c.*-BCCP, *E.coli* BCCP; Ch-ACC, chicken ACCCase; R-ACC, rat ACCCase; Y-ACC, yeast ACCCase. Amino acids identical to tomato MCCase are boxed.

Biotin-dependent carboxylases

All biotin dependent carboxylases catalyze identical first step reactions (Moss and Lane, 1971), as shown in Reaction [1]. The carboxyl group is donated by bicarbonate, new carbon-nitrogen bond formed between biotin and CO₂ at the cost of hydrolysis of ATP. The carboxyl group is subsequently transferred from the carboxybiotinyl intermediate to an acceptor in the second step reaction as shown in Reaction [2]. The overall reaction is given as Reaction [3]:



The final acceptor of the carboxyl group is dependent on the specific enzyme involved.

In mammals there are four biotin-dependent carboxylases. They are ACCases, PCases, PCCases, and MCCases. UCase was found in yeast and unicellular green algae. GCase was found in bacterium *Pseudomonas citronellolis*.

Acetyl-CoA carboxylase (ACCase; EC 6.4.1.2) catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA. This reaction is the first and rate-limiting reaction of fatty acid biosynthesis in bacteria, yeast, animals and plants. Mammalian ACCase is allosterically activated by citrate and isocitrate, which is associated with polymerization of protomer. In addition long-chain fatty acyl-CoA derivatives are inhibitors of ACCases. Affectors of the protomer-

polymer equilibrium determine the level of carboxylase activity. Activation associated with polymerization, while inactivation is the result of disaggregation of the active polymer. cDNAs and genes coding for ACCase have been recently cloned from rat (Lopez-Casillas et al., 1988), chicken (Takai et al., 1987; 1988), yeast (Al-Fell et al., 1992) and *E.coli* (Li and Cronan, 1992a; 1992b; Kondo et al., 1991).

Pyruvate carboxylase (PCase; EC 6.4.1.1) catalyzes the carboxylation of pyruvate to form oxaloacetate. It is involved in both gluconeogenesis, lipid biosynthesis and an anaplerotic reaction for the tricarboxylic acid cycle. PCases from animal and yeast are allosterically activated by acetyl-CoA. The amino acid sequences of PCase from rat (Lim et al., 1988), human (Freytag and Collier, 1984) and mouse (Zhang et al., 1993) have been elucidated.

Propionyl-CoA carboxylase (PCCase; EC 6.4.1.3) catalyzes the carboxylation of propionyl-CoA to form methylmalonyl-CoA. In animals and bacteria, the methylmalonyl-CoA is then converted to succinyl-CoA, by vitamin B₁₂-dependent isomerase. The succinyl-CoA can be further oxidized through the tricarboxylic acid cycle. PPCase is involved in the catabolism of certain amino acids, isoleucine, valine, methionine and threonine, and also the catabolism of odd-chain fatty acids. The amino acid sequences of PPCase from rat (Browner et al., 1989; Kraus et al., 1986) and human (Lamhonwah et al., 1986; 1987) have been elucidated from their cDNA sequences.

β -Methylcrotonyl-CoA carboxylase (MCCase; EC 6.4.1.4) catalyzes the carboxylation of β -methylcrotonyl-CoA to form β -

methylglutaconyl-CoA. The β -methylcrotonyl-CoA comes from the degradation of the amino acid leucine. The β -methylglutaconyl-CoA is further oxidized into acetyl-CoA and eventually CO₂ in the tricarboxylic acid cycle. Unlike ACCase, PCCase and PCase, genes or cDNAs coding for MCCase has never been cloned before from any organism. Therefore the primary sequence of this enzyme is still unknown.

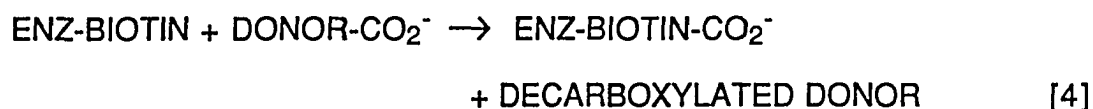
Geranyl-CoA Carboxylase (GCCase; EC 6.4.1.5) catalyzes the carboxylation of geranyl-CoA to form carboxygeranyl-CoA. It plays central role in the bacterial degradation of isoprenoid compounds (Seubert and Remberger, 1963). It has been found in *Pseudomonas citronellolis* (Seubert et al., 1978), *Pseudomonas aeruginosa*, *Pseudomonas mendocina* and *Acinetobacter species* (Cantwell et al., 1963). In *Pseudomonas citronellolis*, the enzyme is inducible by citronellol (Seubert et al., 1978). The carboxylase also catalyzes the carboxylation of farnesyl-CoA and β -methylcrotonyl-CoA.

Urea carboxylase (UCase) catalyzes the carboxylation of urea to form allophanate. It was found in certain yeast and unicellular green algae when urea is their sole nitrogen source (Roon and Levenberg, 1969; Domnas, 1962). The gene coding for UCCase from yeast was recently isolated from yeast (Genbauffe and Cooper, 1991).

Biotin-dependent decarboxylases

Biotin-dependent decarboxylases catalyze reactions that can be considered as the reverse of the reactions catalyzed by biotin dependent-carboxylases. The energy released by the decarboxylation

reaction is utilized to couple the transportation of Na^+ out of cells of certain anaerobic microorganisms. All biotin-dependent decarboxylases catalyze a first step reaction in which biotin is carboxylated as shown in Reaction [4]. The carboxyl group is donated by the specific-donor depending on the enzyme. The carboxyl group is subsequently released from the carboxybiotinyl intermediate as shown in Reaction [5]. The overall reaction is given as Reaction [6]:

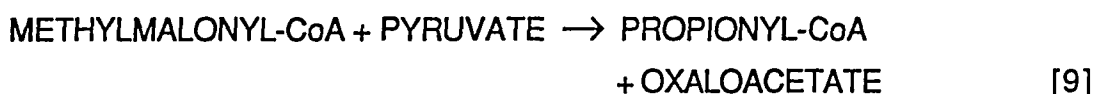
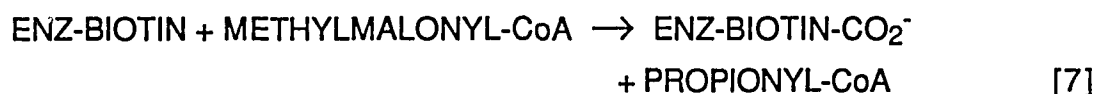


Methylmalonyl-CoA Decarboxylase catalyzes the decarboxylation of methylmalonyl-CoA to produce propionyl-CoA and CO_2 . This reaction is the terminal step in lactate fermentation in *Micrococcus lactilyticus* (Galivan and Allen, 1967; 1968).

Oxaloacetate decarboxylase (OADCase; E.C. 4.1.1.3) catalyzes the decarboxylation of oxaloacetate to produce pyruvate and CO_2 . The enzyme is found during citrate fermentation (Stern, 1967). The genes coding for this enzyme have been recently isolated from *Salmonella* (Woehlke et al., 1992) and *Klebsiella* (Schwarz and Oesterhelt, 1985; Schwarz et al., 1988; Laußermair et al., 1989).

Biotin-dependent transcarboxylase

Methylmalonyl-CoA:pyruvate transcarboxylase (TCase; EC 2.1.3.1) is the only known biotin-dependent transcarboxylase. It catalyzes the transfer of carboxyl group from methylmalonyl-CoA to pyruvate, producing propionyl-CoA and oxaloacetate. The two two-step reactions are shown below in Reactions [7] and [8]. The overall reaction is given in reaction [9].



The enzyme was found in *Propionibacteria* (Werkman and Wood, 1942), has been purified to homogeneity and studied extensively in Wood's laboratory (Wood et al., 1963). The reaction is the terminal reaction in the fermentation of glucose, glycerol and lactate to produce propionate as the end-product. Recently, the genes coding for this enzyme have been isolated (Murtif et al., 1985; Samols et al., 1988).

β -Methylcrotonyl-CoA Carboxylase

MCCases have been investigated from bacteria and animals (Schiele and Lynen, 1981; Fall, 1981; Lau and Fall, 1981a). They have been highly purified and well characterized from *Achromobacter*,

Pseudomonas citronellis and bovine kidney. In each case, the enzyme is composed with two different subunits. The large subunit has a molecular weight of 73,000 to 96,000 and biotin attached. The small subunit has a molecular weight of 61,000 to 78,000 and is biotin-free. The molecular weight of the holoenzyme is between 520,000 and 835,000. The enzyme activity was also found in *Mycobacterium* (Lynen et al., 1961); human fibroblast (Wolf and Kalousek, 1978), human placenta (Lau and Fall, 1979); bovine liver, adrenals, lung, skeletal muscle; rat kidney, liver (Lau and Fall, 1981); somatic carrot embryos, rape seed, sunflower seed, maize leaf and carrot leaf (Wurtele and Nikolau, 1990); green pea leaves (Baldet et al., 1992).

Compared to the extensive characterizations of MCCase from animal and bacterial sources, knowledge of plant MCCase is limited. MCCase activity was only recently found in plant systems (Wurtele and Nikolau, 1990). The finding that MCCase is the mitochondria of plant cells (Baldet et al., 1992; Wurtele et al., in preparation) suggests that plant MCCase may function in the catabolism of leucine as in animals and bacterials (Fig. 3). In addition, MCCase may have a metabolic function in the "mevalonate shunt" by which mevalonate can be metabolized to non-isoprenoid compound.

Unlike the cases of ACCase, PCCase, PCase, UCase, OADCase and TCase, neither the gene nor the cDNA coding for this enzyme has been isolated from any organism.

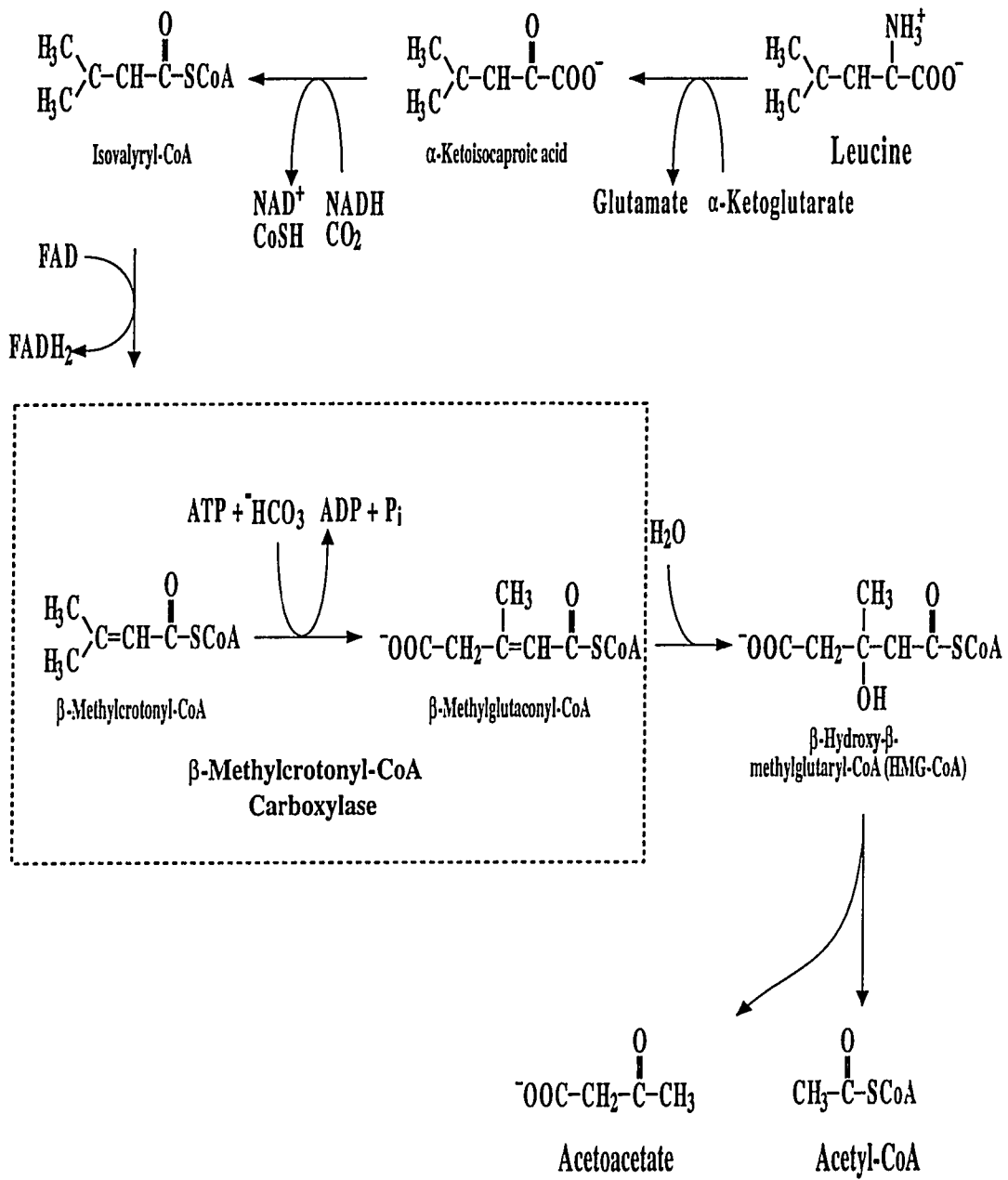


Figure 3. Catabolism of leucine in animal and bacterial systems.

Structure of β -methylcrotonyl-CoA carboxylase **β -Methylcrotonyl-CoA carboxylase from *Achromobacter***

MCCase has been highly purified by Lynen's laboratory (Himes et al., 1963; Apitz-Castro et al., 1970). It has a molecular weight of 760,000 and is composed of two different polypeptides. The large polypeptide (B-subunit) has a molecular weight of 96,000 whereas the small one (A-subunit) has a molecular weight of 78,000. This leads to a assumed MCCase structure of A_4B_4 . The large subunit contains biotin prosthetic group.

The enzyme has the ability to carboxylate free biotin (Himes et al., 1963). Based on this property, Lynen established the two-step reaction mechanism of biotin enzymes via the carboxybiotin intermediate (Lynen et al., 1961; Knappe et al., 1961). The carboxylation site on biotin was identified as the N-1 position (Knappe et al., 1963). Later this was confirmed by several other laboratories with other biotin-containing enzymes (Moss and Lane, 1971). The biotin prosthetic group was shown to be bound to the ϵ -amino group of a lysine residue (Knappe and Beiderbick et al., 1962; Knappe and Wenger et al., 1963) which has also been the case in all the other biotin-containing enzymes investigated (Moss and Lane, 1971). When bacteria are grown in the absence of biotin, approximately 20% of the MCCase is in the apoenzyme form. With sufficient exogenously supplied biotin, all the MCCase is in the holoenzyme form (Höpner and Knappe, 1965).

There are observations of dissociation of the enzyme complex. The dissociation is promoted by iodoacetamide (Schiele and Sturzer, 1975). The substrate β -methylcrotonyl-CoA decreases the degree of dissociation of the enzyme, but ATP has no influence on the dissociation.

The isolated biotin-containing subunit is able to catalyze the carboxylation of free biotin (Schiele et al., 1975). Thus the biotin-containing subunit has both BCCP function and biotin carboxylase function. Transfer of the carboxyl group from β -methylglutaconyl-CoA to free biotin, in the presence of the biotin-free subunit, was not demonstrated. But when both subunits were incubated together, the overall enzymatic activity was regenerated. Based on this observation, the biotin-free subunit was assumed to carry carboxyltransferase function.

β -Methylcrotonyl-CoA carboxylase from *Pseudomonas citronellolis*

β -Methylcrotonyl-CoA carboxylase from *Pseudomonas citronellolis* was purified and characterized in Fall's laboratory (Fall and Hector, 1977; Hector and Fall, 1976). MCCase in this organism is inducible by growing the cells in the presence of leucine or isovalerate as the sole carbon source. The molecular weight of this enzyme is between 520,000-580,000 as measured by gel filtration and from exclusion limit polyacrylamide gel electrophoresis. The enzyme is composed of two types of subunits. The molecular weights of the two subunits are 73,000 and 63,000. The biotin is bound to the large subunit.

β -Methylcrotonyl-CoA carboxylase from bovine kidney

β -Methylcrotonyl-CoA carboxylase from bovine kidney was also purified and characterized in Fall's laboratory (Lau et al., 1979; Lau et al., 1980; Lau and Fall, 1981b). The molecular weight of the enzyme was estimated as 835,000. Two nonidentical subunits have molecular weights of 73,500 and 61,000, respectively. The large subunit contains the biotin prosthetic group.

The enzyme was found in mitochondria (Hector et al., 1980). It appears to be associated with the inner mitochondrial membrane. Release of MCCase from the inner membrane fraction was achieved by polyethylene glycol treatment.

Metabolic role of β -methylcrotonyl-CoA carboxylase

The role of MCCase in the catabolism of leucine has been well established in bacterial and animal systems (Moss and Lane, 1971). Isotopically labeled leucine was initially used to elucidate the pathway of leucine degradation. The degradation products of leucine were found to be acetoacetate (Zabin and Bloch, 1950; Coon, 1950) and acetate (Coon and Gurin, 1949). Radioactive label from ^{14}C -Bicarbonate was incorporated into the final product acetoacetate during leucine catabolism (Plaut, 1951; Plaut and Lardy, 1951). Based on these data, Coon first proposed the entire leucine degradation pathway (Coon et al., 1955). In this pathway (Figure 4), leucine is converted to α -ketoisocaproate by a transamination reaction and then to a CoA

thioester isovaleryl-CoA. The isovaleryl-CoA is subsequently dehydrogenated to form β -methylcrotonyl-CoA. The action of MCCase convertes β -methylcrotonyl-CoA to β -methylglutaconyl-CoA, which is further hydrated by a specific hydratase to produce β -hydroxyl- β -methylglutaryl-CoA (HMG-CoA). The final reaction is the cleavage of HMG-CoA to form acetoacetate and acetyl-CoA.

The leucine catabolism in plants has not been established. This is partially due to the fact that there are no clear data to show the presence of methylglutaconyl-CoA hydratase in plants (Yu-Ito et al., 1982). Also the existence of MCCase in plants was not known until very recently (Wurtele and Nikolau, 1990; Baldet et al., 1992). The discovery of MCCase in plant kingdom along with other indirect evidence (Koops et al., 1991; Overton, 1985) suggests that in plants leucine is catabolized through the same pathway as in animal and bacteria.

Gerbling and Gerhardt (1989) have provided evidence *in vitro* that leucine can be catabolized by an alternative pathway which may be located in peroxisomes. β -Methylcrotonyl-CoA, an intermediate from leucine degradation, is proposed to be converted isobutyryl-CoA, which is further converted to propionyl-CoA and subsequently acetyl-CoA. Thus, in this pathway, MCCase is not required for leucine catabolism. However, there is no report of *in vivo* evidence for the peroxisome leucine degradation pathway.

Studies with animals have shown that mevalonate can be diverted from isoprenoid biosynthetic pathway and used for the synthesis of

non-isoprenoid compound via a hypothetical pathway "mevalonate shunt" (Edmond and Popjak, 1974). MCCase catalyzes a central reaction in "mevalonate shunt". Nes and Bach (1985) demonstrated that in primary leaf tissue of wheat seedlings [^{23}H]mevalonate was incorporated into long-chain fatty alcohols as efficiently as into sterols, whereas [^{53}H]mevalonate was metabolized only to sterol. These results suggest that a similar pathway may be operating in higher plants.

Plant Biotin-Containing Enzymes

Knowledge of biotin-containing enzymes is mostly from animal and microbial sources (Dakshinamurti et al., 1985; Wood and Barden, 1977; Moss and Lane, 1971). Recently, molecular cloning of genes coding for ACCase (Takai et al., 1987; Rylatt and Wallace, 1971; Kondo et al., 1991; Li and Cronan, 1992), PCase (Freytag and Collier, 1984; Alberts and Vagelos, 1972), PCCase (Lamhonwah et al., 1986; 1987), TCcase (Murtif et al., 1985), and OADCcase (Alberts and Vagelos, 1972; Woehlke et al., 1992) has enabled the elucidation of the primary sequence of these enzymes. In contrast to the extensive characterization of biotin-containing enzymes from animal and microbial sources, knowledge of biotin-containing enzymes from plants is limited. Indeed, until recently the only biotin-containing enzyme that was known in plants was ACCase (Wurtele and Nikolau, 1990). However, there are indications that additional biotin-containing polypeptides occur in extracts from plants (Nikolau et al., 1987; 1984a; 1984b; 1985;

Wurtele and Nikolau, 1992; Nikolau et al., 1992; Kannangara and Jensen, 1975; Hoffman et al., 1987; Baldet et al., 1992;1993). Recently, the occurrence of three additional biotin-containing enzyme activities have been reported in plants (Wurtele and Nikolau, 1990; 1992), these being PCCase, PCase and MCCase. However, the identification of each enzyme activity with biotin-containing polypeptides is still unclear. In this dissertation I present evidence for identification of the 78 kD biotin-containing polypeptide of tomato as the biotin-containing subunit of MCCase.

The best characterized biotin-containing enzyme in plants is ACCase. Plant ACCase has been reported to be of approximately 500 kD. Most of plant ACCases have been shown to be composed with two subunits of 220,000 (Egin-Buhler and Ebel, 1983; Slabas and Hellyer, 1985; Charles and Cherry, 1986; Hellyer et al., 1986; Slabas et al., 1986; Egli et al., 1992;1993). ACCase with biotin-containing subunits of 60,000 were reported from maize (Nikolau and Hawke, 1984) and somatic carrot embryo (Nikolau et al., submitted). The reports of two types of ACCases in plants suggest that these organisms contain isoenzyme of ACCase. In plants, malonyl-CoA is an intermediate involved in multiple processes including the biosynthesis of fatty acids (Stumpf, 1987), very long-chain fatty acids (Kolattukudy et al., 1976; Bessoule et al., 1989), flavonoids (Hahlbrock, 1981), stilbenoids (Gorham, 1980), anthroquinones (Packter, 1980), malonyl derivatives of D-amino acids (Ogawa et al., 1973; Liu et al., 1983), malonation of 1-aminocyclopropane carboxylic acid (Liu et al., 1983; Su et al., 1985)

and malonic acid (Stumpf and Burris, 1981). Thus ACCase isoenzymes may be located in different compartments in the plant cell or expressed at different developmental stage of the plant's life for these multiple metabolic processes.

The discovery of MCCase activity in plant protein extracts was recently reported (Wurtele and Nikolau, 1990). The enzyme appears to be biotin-dependent since the activity is avidin-sensitive. The existence of MCCase in plants was confirmed by this dissertation and other reports (Chen et al., 1993; Baldet et al., 1992; Song, in preparation; Diez, in preparation). In addition, a cDNA, originally isolated from tomato (Hoffman et al., 1987), as coding for a putative plant biotin enzyme has been shown in this dissertation to be a cDNA coding for the biotin subunit MCCase.

Holocarboxylase Synthetase

Biotin is covalently attached to biotin-containing enzymes via an amide linkage between the carboxylic acid group of biotin and the ϵ -amino group of a lysine residue of the enzyme. The biochemistry of the attachment of biotin to apoenzyme has been extensively studied in animal and bacterial systems (Moss and Lane, 1971). In 1960's, several laboratories found that when organisms are deprived of biotin activities of biotin-containing enzymes decrease. When the biotin-deficient cells or cell-free extracts were incubated with free biotin, Mg^{2+} and ATP, the activities of biotin-containing enzymes were rapidly

restored (Kosow and Lane, 1961a; 1961b; 1962; Kosow et al., 1962; Jacobs and Majerus, 1970; Deodhar and Mistry, 1969a; 1969b). It has been demonstrated that the rapid restoration of enzyme activities is independent of new protein synthesis (Deodhar and Mistry, 1969a; 1969c). Therefore under the biotin deficiency condition, the total enzyme (apoenzyme plus holoenzyme) concentration remains the same level as under sufficient biotin condition (Kosow and Lane, 1961a).

The attachment of biotin to apoenzymes was found to be catalyzed by holocarboxylase synthetase (Kosow and Lane, 1961a; 1962a; 1962b; 1962c). Utilizing methylmalonyl-CoA:pyruvate apotranscarboxylase as a model, Lane concluded that holocarboxylase synthetase catalyzed a two-step Reaction [10] and [11], (Lane et al., 1964a; 1964b). The overall reaction is given as Reaction [12]:



The enzyme has very broad specificity toward the acceptor apoenzymes from different sources. It has been demonstrated that holoenzyme synthetase from *Propionibacterium shermanii* is able to catalyze biotinylation of apopropionyl-CoA carboxylase from rat, apo- β -methylcrotonyl-CoA carboxylase from *Achromobacter* (McAllister and Coon, 1966). Consequently, the amino acid sequences around

biotinylation sites in biotin enzymes are conserved during evolution (Figure 2). The broad specificity toward apoenzymes by holoenzyme synthetase was also demonstrated by the fact when DNA sequences coding for biotin-containing enzymes are expressed in a heterologous host, the gene product is biotinylated by the host holocarboxylase synthetase (Hoffman et al., 1987).

Although the holoenzyme synthetase from animal and bacteria have been studied to some extent, current knowledge of the biochemistry of biotinylation in plants is very limited. Indeed this dissertation reports for the first time that biotinylation of the apoenzyme is an important mechanism for regulating the organ-specific activity of MCCase in plants.

Explanation of Dissertation Format

This dissertation contains a general introduction to the problem addressed with a critical review of the literature. The introduction is followed by three research papers as manuscripts, after which there is a general summary and conclusions. Finally, the literature cited in the general introduction and the general summary and conclusions is given.

**PAPER I. MOLECULAR CLONING OF cDNAS AND GENES
 CODING FOR β -METHYLCROTONYL-CoA
 CARBOXYLASE OF TOMATO**

**MOLECULAR CLONING OF cDNAS AND GENES CODING FOR β -
METHYLCROTONYL-CoA CARBOXYLASE OF TOMATO**

**Xun Wang¹, Eve Syrkin Wurtele², Greg Keller^{1,*}, Angela L.
McKean¹, Basil J. Nikolau^{1,†}**

¹Department of Biochemistry and Biophysics, and ²Department of
Botany, Iowa State University, Ames, Iowa 50011

†Corresponding Author: Dr. Basil J. Nikolau
Department of Biochemistry & Biophysics,
Iowa State University,
Ames, IA 50011
Phone: (515) 294-9423
FAX: (515) 294-0453

*Present Address: Agracetus,
8520 University Green,
Middleton, Wisconsin.

Running Title: β -Methylcrotonyl-CoA Carboxylase

¹Abbreviations:

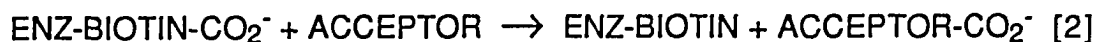
ACCase, acetyl-CoA carboxylase; BSA, bovine serum albumin; EtBr, ethidium bromide; HEPES, N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; IPTG, isopropyl- β -D-thiogalactopyranoside; MCCase, β -methylcrotonyl-CoA carboxylase; NBRF, National Biomedical Research Foundation; PAGE, polyacrylamide gel electrophoresis; PCase, pyruvate carboxylase; PCCase, propionyl-CoA carboxylase; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TCase, methylmalonyl-CoA:pyruvate transcarboxylase; Tris-HCl, tris[hydroxymethyl]aminomethane hydrochloride

ABSTRACT

Biotin-containing enzymes catalyze critical reactions in diverse metabolic processes. In contrast to the extensive characterization of biotin-containing enzymes from animal and microbial sources, little is known about these enzymes from plants. Hoffman et al. serendipitously isolated a cDNA from tomato that appears to code for a biotin-containing polypeptide. This cDNA was used as probe to isolate three longer cDNAs (1.0 kb, 1.5 kb, and 1.7 kb) and two genomic clones from tomato. These longer cDNA clones have been expressed in *Escherichia coli* and the expressed proteins were purified to homogeneity. The antisera against these purified proteins specifically inhibit β -methylcrotonyl-CoA carboxylase activity in tomato cell free extracts. Thus, these cDNAs are identified to code for a biotin-containing polypeptide that is a subunit of β -methylcrotonyl-CoA carboxylase, an enzyme catalyzing a central reaction of leucine catabolism. Western blot analysis indicates that the biotin-containing subunit of tomato β -methylcrotonyl-CoA carboxylase is a 78 kDa polypeptide. Northern blot analysis identified the 2.4 kb mRNA for the biotin-containing subunit of β -methylcrotonyl-CoA carboxylase. Comparison of the amino acid sequence of the biotin-containing subunit of β -methylcrotonyl-CoA carboxylase deduced from the nucleotide sequences of the cDNAs, with other biotin-containing enzymes suggests that this protein carries the biotin carboxylase and the biotin carboxyl carrier functions.

INTRODUCTION

Biotin is an essential co-factor for a set of enzymes involved in diverse metabolic processes, such as lipid metabolism, amino acid metabolism and carbohydrate metabolism (Moss and Lane, 1971; Wood and Barden, 1977; Wood and Kumar, 1985; Dakshinamurti and Bhagavan, 1985; Knowles, 1989; Mildvan et al., 1991). Although each enzyme has distinct metabolic functions, all biotin enzymes share the same catalytic mechanism. The biotin prosthetic group in these enzymes serves as an intermediate carrier of the carboxyl group being transferred from one substrate to another (Moss and Lane, 1971; Wood and Barden, 1977; Knowles, 1989). The overall reactions catalyzed by these enzymes are carried out in two steps. The first step involves the carboxylation of the enzyme-bound biotin by the biotin carboxylase activity. In the second step, the carboxyl group is transferred from carboxy-biotinyl enzyme intermediate to an appropriate acceptor by the carboxyltransferase activity. For biotin-containing carboxylases, the two-step Reactions, [1] and [2], result in the overall Reaction [3]:



Knowledge of biotin-containing enzymes has been gathered mostly from the animal and microbial kingdoms (Dakshinamurti et al., 1985;

Wood and Barden, 1977; Moss and Lane, 1971). The biochemistry of biotin-containing enzymes from these sources has been well established. Recently, molecular cloning of genes or cDNAs coding for biotin-containing enzymes from these systems has enabled the elucidation of the primary sequences of biotin-containing enzymes. Examples of such characterizations are the molecular cloning of sequences coding for ACCase¹ (Li and Cronan, 1992a, b; Kondo et al., 1991; Lopez-Casillas et al., 1988; Takai et al., 1987; 1988; Al-Feel et al., 1992), PCase (Zhang et al., 1993; Lim et al., 1988; Freytag and Collier, 1984), PPCase (Browner et al., 1989; Lamhonwah et al., 1986; 1987; Kraus et al., 1986), TCase (Murtif et al., 1985), oxaloacetate decarboxylase (Woehlke et al., 1992; Schwarz and Oesterheld, 1985; Schwarz et al., 1988; Laußermair et al., 1989) and urea amidolyase (Genbauffe and Cooper, 1991).

In contrast, knowledge of biotin-containing enzymes from plants is limited. Indeed until recently the only biotin-containing enzyme that was known in plants was ACCase (Wurtele and Nikolau, 1990). However, additional biotin-containing polypeptides have been detected in extracts from plants (Nikolau et al., 1987; 1984a; 1984b; 1985; Wurtele and Nikolau, 1992; Nikolau et al., 1992; Kannangara and Jensen, 1975; Hoffman et al., 1987; Baldet et al., 1992; 1993). And in addition to ACCase three other biotin-containing enzyme activities have been reported in plants, these being PCCase, PCase and MCCase (Wurtele and Nikolau, 1990; 1992). However, the identity of the biotin-containing polypeptide associated with each of these activities is still not clear.

In light of the poor understanding of the plant biotin-containing proteins, the present study was undertaken, that resulted in the identification of a cDNA clone coding for the biotin-containing subunit of MCCase from tomato.

MATERIALS AND METHODS

Materials — All restriction endonucleases and DNA modifying enzymes were purchased from Bethesda Research Laboratories, United States Biochemicals, or Promega-Biotechnology, and were used as suggested by the suppliers. The radioisotopes were purchased from Amersham or ICN Biomedicals. All other reagents and biochemicals were purchased from Sigma Chemical Co. MO, United States Biochemicals, or Fisher Scientific.

Tomato (*Lycopersicum esculantus* var. *Rutgers*) were grown in a greenhouse at 18-22 °C under supplemented illumination, with a 15h day length. Plants were watered daily and fertilized at weekly intervals. Leaves were harvested 60-80 days after planting.

Isolation of cDNAs — Two different cDNA libraries were employed. The first (a gift from Dr. G. T. King, Native Plants Inc. Utah) was a λ gt 10 cDNA library prepared by oligo (dT)-priming of mRNA isolated from tomato leaves (King et al., 1988). Approximately 2×10^5 plaque-forming units were subjected to hybridization with a 384 bp tomato cDNA clone (we call TBP), (Hoffman et al., 1987). Seventeen hybridizing clones were detected under stringent hybridization and washing conditions. These clones could be classified into two groups according to the sizes of the cDNA inserts. A representative clone from each of these two classes was chosen and subcloned into pUC19. The resulting

plasmids were called pGKBT (1.5 kb cDNA insert) and pBTN (1.0 kb cDNA insert).

The second cDNA library (a kind gift from Dr. A. Bennett, University of California, Davis) was a pBluescript SK cDNA library, prepared by oligo (dT)-priming of mRNA isolated from ripening fruits of tomato. This library was probed with the 5' most end, *EcoRI-RsaI* fragment from pGKBT (named BX1. see Figure 1 for the restriction map of the GKBT cDNA). From 2.2×10^5 recombinant clones, a single clone was detected under stringent hybridization and washing conditions. The cDNA insert (1.7 kb in length) was subclone into pBluescript SK, and the resulting plasmid was called pMCC-1.

Isolation of Genomic Clones — A tomato genomic library, in Charon 34 phage, was a gift of Dr. G. T. King, Native Plants Inc., Utah. Approximately 2×10^6 recombinant phage were subjected to hybridization with the cDNA clone from pTBP. Two hybridizing clones, λ TG1 and λ TG12, were isolated.

Isolation and Analysis of DNA — Tomato genomic DNA was prepared from plant tissues as described (Dellaporta et al., 1983). Isolation of plasmid DNA and bacteria phage DNA, and manipulation of the isolated DNAs, and transformation of *E. coli* with plasmids were as described by Sambrook et al. (1989). Isolated DNA was digested with restriction endonucleases and the resulting DNA fragments were separated by electrophoresis in 0.7%-1% agarose gels and transferred by capillary action (Southern, 1975), to nylon membranes (MSI), using 25 mM sodium

phosphate buffer (pH 6.5). Southern blots were subjected to hybridization at 65 °C for 12 hours in a 10 ml solution composed of 0.75 M NaCl, 50 mM Tris-HCl (pH 8.0), 10% (w/v) dextran sulfate, 1X Denhardt's solution (50 X Denhardt's solution = 1 g Ficoll, 1 g polyvinylpyrrolidone, and 1 g BSA in 100 ml H₂O), 0.2% (w/v) SDS, 10 mM EDTA, 100 µg/ml of sheared salmon sperm DNA, and 5 ng/ml of ³²P-labeled DNA probe (specific radioactivity >1 X 10⁸ cpm/µg). Following hybridization the blots were washed and the final most stringent wash was with 0.1 X SSC (1X SSC is 0.15 M NaCl, 15 mM sodium citrate), 0.5% (w/v) SDS at 68 °C for 30 min.

DNA restriction fragments, fractionated by electrophoresis in agarose gels were purified by adsorption to powdered glass particles (GeneClean, Bio101), which was based on the method developed by Vogelstein and Gillespie (1979).

³²P-Labeled DNA probes were generated *in vitro* by random-primer supported synthesis of DNA with the Klenow fragment of DNA polymerase I, in the presence of [α^{32} P]-dCTP (Feinberg et al., 1984).

Restriction sites were mapped on DNA fragments from the ladder of partial digestion products and confirmed by complete digestion of the DNA fragment with restriction enzymes, singly and in combination.

Isolation and Analysis of RNA — Total cellular RNA was isolated from tomato leaves and roots, as described by Logemann et al. (1987). RNA was fractionated by electrophoresis in 1.2% (w/v) agarose gel containing formaldehyde as described (Lehrach et al., 1977). The fractionated RNA was transferred to a nylon membrane (MSI) with 10 X

SSC, and the resulting blot was subjected to hybridization with ^{32}P -labeled DNA probe. The hybridization was carried out at 65 °C in a solution composed of 5X SSPE (1X SSPE is 0.15 M NaCl, 1 mM EDTA, 10 mM sodium phosphate buffer (pH 7.4)), 5X Denhardt's solution, 0.5% (w/v) SDS, 100 µg denatured, sheared salmon sperm DNA, 10% (w/v) dextran sulfate and 5 ng/ml of ^{32}P -labelled DNA probe. After hybridization the blots were washed three times with 0.1X SSC, 0.5% SDS at 65 °C.

DNA Sequencing — cDNAs were cloned in Bluescript SK vector (Stratagene) in both potential orientations. Unidirectional deletions were generated by the procedure developed by Henikoff (1984). Single-stranded templates of the deletion mutant constructs were prepared as described (Viera and Messing, 1987). DNA sequencing was carried out using the Sequenase II kit purchased from United States Biochemical Corp., which is based on the dideoxy-mediated chain-termination procedure developed by Sanger (1977).

Computer Analysis of Nucleotide and Predicted Amino Acid Sequence — All computer-assisted analyses of nucleotide and predicted amino acid sequences were performed utilizing the sequence analysis software package of the University of Wisconsin Genetics Computer Group (GCG; Madison, WI).

Expression of Proteins in E. coli — *E. coli* JM101 harboring expression vectors was grown overnight at 37 °C in 10 ml LB media containing the appropriate antibiotic. The overnight culture was added

to 1 liter of fresh LB media supplemented with antibiotic. After four hours of growth, expression of the fusion protein was induced by the addition of IPTG to a final concentration of 1 mM. Four hours after induction with IPTG, cells were collected by centrifugation at 2,000 *g* for 10 min and washed twice with 50 mM Tris-HCl (pH 7.0). The resulting *E. coli* pellet was resuspended in 5 ml of 50 mM Tris-HCl (pH 7.0), 1 mM EDTA, 2% (w/v) SDS and then incubated at 100 °C in a bath of boiling water for 15 min. The mixture was diluted ten-fold with 50 mM Tris-HCl (pH 7.0), 1 mM EDTA before passing through a 2.5 ml avidin-agarose affinity column (Pierce, IL). The column was washed with 250 ml of 50 mM Tris-HCl (pH 7.0), 1mM EDTA, 0.2% (w/v) SDS. Biotin-containing polypeptides were eluted from the column with 3 ml of 100 mM Tris-HCl (pH 7.0), 1 mM EDTA, 2% (w/v) SDS buffer, and the column was sealed at bottom and heated to 100 °C in a bath of boiling water for 20 min. The eluate was collected from the column by centrifugation at 4,000 *g* for 10 min.

Production of Polyclonal Antibodies — Avidin affinity purified, expressed fusion proteins were fractionated by SDS-PAGE. After the gels were stained with Coomassie Brilliant Blue, protein bands were excised from the gel. Gel slices which contained about 200 µg of protein were mixed with Freund's Complete Adjuvant and macerated by passing through a syringe. The emulsified antigens were injected at 24-36 sites distributed over the back of New Zealand White female rabbits. Four weeks latter, the animals were challenged at two week intervals by intramuscular injection into each of the two hind limbs of

the animals with freshly prepared antigen emulsified in Freund's Incomplete Adjuvant. Blood was collected from such rabbits and allowed to coagulate. After centrifugation at 4,000g for 10 min, serum was collected, adjusted to 0.02% (w/v) NaN_3 and stored at -20 °C.

SDS-PAGE and Western Blot Analysis — SDS-PAGE was carried out as described by Laemmli et al. (1970). Proteins were transferred from the gel to nitrocellulose filters with a semi-dry trans blot apparatus according to the manufacturer's instructions (PolyBlot, American Bionetics, Inc.). Immunological detection of proteins was conducted with antiserum diluted between 1:200 to 1:1000 in a 3% (w/v) BSA solution, following which, antigen-antibody complexes were detected with ^{125}I -protein A. Biotin-containing polypeptides were detected with ^{125}I -streptavidin (Nikolau et al., 1985).

Purification of Biotin-Containing Polypeptides from Plants — Expanding tomato leaves were harvested and frozen with liquid nitrogen, and while still frozen the tissue was ground to a powder with a mortar and pestle. Two volumes of SDS-containing extracting buffer [100 mM Tris-HCl (pH 7.0), 10 mM DTT, 5 mM EDTA, 2% (w/v) SDS] was added to the powder. After additional homogenization with the pestle, the mixture was transferred to a glass tube and heated at 100 °C in a bath of boiling water for 15 min. The biotin-containing polypeptides were isolated with avidin-agarose affinity chromatography by the procedure described in the isolation of the expressed proteins from *E. coli* extracts.

Enzyme Extraction — Expanding tomato leaves were harvested, frozen with liquid nitrogen, and ground to a powder in a mortar and pestle. Two volumes of extraction buffer [100 mM HEPES-KOH (pH 7.0), 1 mM EDTA, 20 mM 2-mercaptoethanol, 100 µg/ml PMSF, 20% (v/v) glycerol, 0.1% (v/v) Triton X-100] was added to the powder, and the mixture was homogenized further with the pestle. The mixture was filtered through four layers of cheesecloth and the filtrate was centrifuged at 10,000*g* for 10 min. Low molecular weight molecules were removed from the supernatant by passing 1 ml of such an extract through a 5 ml Bio-Gel P-6DG gel filtration column.

Protein concentration was determined by the Bradford method (Bradford, 1976).

Enzyme Assays — Biotin dependent carboxylase activities were measured as the rate of incorporation of radioactivity from $\text{NaH}^{14}\text{CO}_3$ into the acid-stable products (Wurtele and Nikolau, 1990).

RESULTS

The biotin prosthetic group is always attached to biotin enzymes via an amide bond between the carboxyl group of biotin and the ϵ -amino group of a lysine residue (Moss and Lane, 1971). The structural features that define the lysine residue for biotinylation are still not clear, although they appear to have been highly conserved during evolution (Samols et al., 1988). The primary sequence flanking the lysine residue targeted for biotinylation is fairly well conserved in all biotin enzymes; that sequence is (Ala/Val)-Met-Lys-(Met/Ala). However, this sequence is not the only structural information that defines the biotinylation site, since mutations away from this sequence motif affects biotinylation of this lysine residue (Murtif and Samols, 1987).

A consequence of the high degree of conservation in the site of biotinylation is that when a DNA sequence coding for a biotin enzyme is expressed in a heterologous host, the protein product can be biotinylated (Hoffman et al., 1987; Murtif and Samols, 1987; Cronan, 1990; Collins, et al., 1987). Indeed such biotinylation events have been reported as artifacts of immunological screening of expression cDNA libraries utilizing the "biotin-avidin" system for detection (Hoffman et al., 1987; Collins, et al., 1987). One example of these was the result of an immunological search of a tomato leaf cDNA expression library for clones coding for chloroplast membrane proteins, which resulted in the isolation of a 384 bp cDNA clone what we call TBP (Hoffman et al.,

1987). This clone contains an incomplete translational open-reading frame of 70 amino acids that shows sequence similarity to biotin enzymes, including the sequence Ala-Met-Lys-Met. The most striking sequence similarity is the similarity to the TCase of *Propionibacterium*.

Due to the lack of structural and metabolic information about biotin enzymes of plants, we undertook the experiments described below to ascertain whether TBP is a clone of an mRNA that codes for a plant biotin enzyme, and to identify that enzyme.

Cloning and Sequencing cDNAs Coding for of a Putative Biotin Enzyme from Tomato — The cDNA clone TBP was initially used to screen a λ gt10 cDNA library prepared from poly-A RNA isolated from tomato leaves. This resulted in the isolation of two cDNA clones of approximately 1.5 kb (named GKBT) and 1.0 kb (named BTN). The cDNA GKBT was initially characterized in terms of a restriction map and subsequently determination of its nucleotide sequence. The cDNA was delineated into four parts by three *RsaI* restriction sites (Figure 1). The three 5'-most portions were subcloned into the *SmaI* site of pUC19 to generate pBX1, pBX2 and pBX3. Double stranded DNA templates from these three plasmids were sequenced with the M13 universal primer and pUC reverse primer (Promega Corp. Madison, WI). In addition, the cDNA GKBT was cloned in the two possible orientations into the *EcoRI* site of pBluescript SK to generate the plasmids pBBT(+) and pBBT(-). A series of nested deletions of these two plasmids were generated with exonuclease III and S1 nuclease. Single stranded templates from these

deleted plasmids were sequenced with the M13 universal primer. The complete strategy for determining the nucleotide sequence and the restriction map of GKBT are illustrated in Figure 1. The sequence of GKBT is presented in Figure 2.

There are 36 adenosine nucleotides at one end of the GKBT sequence. The TBP sequence was located at the same end (nucleotides 1667 to 2095 in Figure 2). Thus, this end of the sequence of GKBT was identified as the 3' end of the corresponding mRNA. The sequence of GKBT extended the TBP translational reading frame for an additional 52 codons (nucleotides 1511 to 1667 in Figure 2). This translational frame contains the putative biotinylation site including the tetrapeptide Ala-Met-Lys-Met (amino acids 399 to 402 in Figure 2). Surprisingly, at least 8 stop codons per frame were found in all three translational reading frames upstream of this region in a range of 483 nucleotides (nucleotide 1027 to 1510 in Figure 2). At the 5'-end of the clone there was a translational open reading frame of 307 bp coding for 102 codons (nucleotides 719 to 1026 in Figure 2).

The lack of a continuous translational open reading frame throughout the sequence was somewhat surprising. However, careful examination of the nucleotide sequence of GKBT revealed that the region of the clone that contained stop codons in all three translational reading frames (nucleotides 1027 to 1510 in Figure 2), was flanked by consensus exon/intron and intron/exon junctions (underlined in the sequences shown in Figure 2), (Padgett et al., 1985). The identification of these intron/exon consensus junction sequences suggested that the

portion of the GKBT sequence that contains stop codons in all three translational reading frames, is potentially an intron, and that the GKBT cDNA may be a clone of a pre-mRNA or incompletely spliced mRNA. Confirmation of this hypothesis was obtained from a number of different experiments.

Northern blots of RNA isolated from tomato leaves were probed with the putative intron sequence (nucleotides 1039 to 1443 in Figure 2), the entire GKBT sequence, and the inserts from pBX1 and pBX3 which are putative exons. The latter three probes all detected a single hybridizing RNA band of about 2.4 kb in size, but the probe specific for the putative intron was unable to detect this RNA band (data not shown). Therefore, the sequence that was putatively identified as an intron does not occur on the same RNA molecule that was detected by the entire GKBT sequence. However, all four probes hybridized to both of the genomic clones isolated with the probe TBP (see later discussion on the isolation of the two genes). These data are consistent with the presence of an intron in the GKBT sequence. The final evidence that the cDNA in GKBT indeed contained an intron came from the complete sequencing of the 1.0 kb cDNA in pBTN.

The strategy that was used to sequence the clone contained in pBTN is summarized in Figure 1. The sequence of BTN was identical to GKBT, except for (1) the additional 72 nucleotides at the 5'-end of the clone and (2) the absence of the putative intron sequence. Indeed the putative intron sequence was "spliced" out of GKBT exactly as predicted, so that in the sequence of BTN nucleotide 967 was juxtaposed to nucleotide

1534 of the GKBT sequence. The elimination of this intron sequence resulted in the identification of a large translational open reading frame that extends from the 5'-end of BTN for a total of 220 codons.

The clone BTN is obviously a partial cDNA clone since it hybridizes to a 2.4 kb transcript on Northern blots (see later in Results). Therefore, an additional cDNA library, which was made from poly A RNA isolated from ripening tomato fruit, was screened with the insert from pBX1, which is the 5'-most end of the GKBT sequence (nucleotide 719 to 1038 in Figure 2). From approximately 2.2×10^5 recombinant plasmids that were subjected to hybridization, a single clone, containing a 1.7 kb cDNA, was isolated; this clone was named MCC-1. The strategy that was used to sequence MCC-1 and the restriction map of this cDNA are shown in Figure 1. The nucleotide sequence of the 3'-end of MCC-1 is identical to the entire sequence of BTN. In addition, MCC-1 is 646 bp longer at its 5'-end. The nucleotide sequence of MCC-1 contains a translational open reading frame that extends from its 5'-end to a termination codon TGA (nucleotide 1877 in Figure 2). This open reading frame codes for a polypeptide of 436 amino acids.

Figure 2 shows the nucleotide sequences of the cDNA TBP, MCC-1, BTN, GKBT and the deduced amino acid sequences from these cDNAs. As discussed above, the 3'-end of the cDNA was identified by the presence of the stretch of adenosine nucleotides. However, the conserved eukaryotic polyadenylation signal 5'-AATAAA-3' (Proudfoot and Brownlee, 1976) is not present in the 260 nucleotides of the 3'-noncoding sequence upstream of the polyA tail. Instead, the sequence

5'-ACTCAAAA-3', a proposed polyadenylation signal sequence found in the soybean proteinase inhibitor cDNA (Hammond et al., 1984) was located 40 nucleotides upstream of the poly A tail. It has been reported that plant polyadenylation signals may diverge significantly from the animal consensus sequence (Hunt et al., 1987).

Comparison of the Predicted Amino Acid Sequence of MCC-1 with Other Biotin Enzymes — A computer search of the NBRF protein data base by using the GCG Package showed that the amino acid sequence predicted from the nucleotide sequence of MCC-1 showed a high degree of similarity to many biotin enzymes so far sequenced. These include PPCase (Browner et al., 1989; Kraus et al., 1986; Lamhonwah et al., 1987; 1989), ACCase (Lopez-Casillas et al., 1988; Takai et al., 1987; 1988; Al-Fell et al., 1992), PCase (Lim et al., 1988; Freytag and Collier, 1984; Zhang et al., 1993), TCase (Murtif et al., 1985), *E. coli* biotin carboxyl carrier protein of ACCase (Rylatt et al., 1977) and the biotin-containing subunits of oxaloacetate decarboxylases (Schwarz and Oesterhelt, 1985; Schwarz et al., 1988; Laußermair et al., 1989; Woehlke et al., 1992). The amino terminal sequence predicted from MCC-1 (MCC-1, 1-193) also shows homology to the *E. coli* biotin carboxylase (Li and Cronan, 1992a; 1992b; Kondo et al., 1991). Figure 3 shows the sequence alignment of rat ACCase, human PPCase, yeast PCase, biotin carboxylase of *E. coli*, biotin carboxyl carrier protein of *E. coli*, *Propionibacterium* 1.3 S subunit of TCase with the amino acid sequence predicted from MCC-1 cDNA clone.

The homology between the MCC-1 sequence and biotin enzymes occurs in two regions, an N-terminal domain (1-191 of MCC-1) and a C-terminal domain (352-436 of MCC-1). In the N-terminal region, the MCC-1 sequence shows approximately 40% amino acid identity with each of the sequences of the biotin enzymes shown in Figure 3. In particular, the similarity between the sequence of this domain with the biotin-carboxylase of *E. coli* suggests that this is the biotin carboxylase functional domain. In the C-terminal region, the MCC-1 sequence shares about 25% amino acid identities with the other biotin enzymes. The conservation between this domain and the biotin-carboxyl carrier protein of *E. coli* and the 1.3 S subunit of TCase of *Propionibacterium* suggests that this is the biotin carboxyl carrier functional domain. These sequence similarities between MCC-1 and other known biotin-containing enzymes confirm the initial identification of the MCC-1 cDNA as coding for a plant biotin-containing enzyme. However, since the similarity between MCC-1 and other known biotin enzymes was not specifically higher for a particular biotin enzyme, we were unable to predict the enzymatic function of the protein coded by MCC-1.

Expression of MCC-1 in E. coli — To generate antibodies to the protein coded by the MCC-1 cDNA, we expressed this sequence in *E. coli*. Two chimeric protein expression vectors were constructed. The cDNA BTN was fused in frame to the 3'-end of the glutathione S-transferase gene in the vector pGEX-2T (Figure 4A). The resulting plasmid, pGEXBTN was introduced into *E. coli*. SDS-PAGE analysis of extracts

from *E. coli* strains harboring pGEXBTN identified the 57 kDa glutathione S-transferase fusion protein (Figure 4B). Western analysis of such gels with ^{125}I -streptavidin revealed that the GEX-BTN fusion protein was biotinylated, as expected (Fig. 4C).

The expression vector pLB was constructed to express the C-terminal portion of MCC-1 as a β -galactosidase fusion protein (Rüther and Müller-Hill, 1983), (Fig. 5A). The chimeric protein expressed from this vector was identified by SDS-PAGE (Fig. 5B) and Western analyses (Figs. 5C and 5D). Western analysis with antibodies to β -galactosidase identified the overexpressed fusion protein as a protein band of about 10 kDa larger than β -galactosidase, which was present in extracts from cells harboring the parent vector pUR289 (Fig. 5C). Western analysis with ^{125}I -streptavidin demonstrated that the β -galactosidase fusion protein was biotinylated (Fig. 5D).

As expected from the originally isolated TBP cDNA, all expressed chimeric proteins were biotinylated in *E. coli*, therefore we were able to purify both expressed fusion proteins to homogeneity by affinity chromatography with immobilized avidin (Fig. 4D for the glutathione S-transferase and Fig. 5E for the β -galactosidase fusion protein). Both purified chimeric proteins were further purified by preparative SDS-PAGE, and used as antigens for the generation of antisera in rabbits.

MCC-1 Codes for the Biotin-Containing Subunit of β -Methylcrotonyl-CoA Carboxylase — The antisera that were generated to the GEX-BTN and LB fusion proteins were utilized to confirm that the cDNA MCC-1 codes for a biotin-containing protein in tomato, and subsequently to

identify the enzymatic function of that biotin protein. Young tomato leaves were harvested as the source for the purification of biotin-containing polypeptides. Proteins were extracted with a buffer containing SDS and the extract was denatured by heating. Identical aliquots of this extract was subjected to affinity chromatography with avidin-agarose columns that were either pretreated with biotin to block biotin-binding sites, or not so pretreated. After extensive washing of the columns to remove nonbinding proteins, the matrices were incubated at 100 °C, in the presence of 2 % SDS. Such harsh conditions were required to elute biotin-containing polypeptides from the column due to the high affinity between avidin and biotin (dissociation constant is 10^{-15} M^{-1} ; Green, 1975). The proteins eluted by this treatment and an aliquot of the initial extract were subjected to SDS-PAGE and Western analysis (Fig. 6). Incubation of the blot with the antiserum against the glutathione S-transferase fusion protein (anti-GEX-BTN) followed by ^{125}I -Protein A revealed a 78 kDa biotin-containing polypeptide in the crude extract (Fig. 6, lane 1). This polypeptide was retained and purified by the nonbiotin-treated avidin affinity column (Fig. 6, lane 2), but was not bound by the avidin affinity column preincubated with biotin. The same result was obtained with the antiserum against β -galactosidase fusion protein (anti-LB; data not shown). Therefore, independent of the glutathione S-transferase or β -galactosidase epitopes that each of these antibodies recognize, respectively, these antibodies identify the identical 78 kDa polypeptide, which because of its tight binding to avidin, we conclude,

contains biotin. These results demonstrate that the MCC-1 cDNA codes for a 78 kDa biotin-containing polypeptide.

Four biotin-dependent enzyme activities are known in the plant kingdom, these being ACCase, PCCase, MCCase and PCase (Nikolau and Wurtele, 1990). To determine which of these, if any, are associated with the 78 kDa biotin-containing polypeptide, the anti-GEX-BTN serum was used in the experiment illustrated in Fig. 7. Tomato leaf enzyme extracts were incubated, on ice, for 40 minutes with increasing amount of either the antiserum or the preimmune serum. The resulting mixtures were subsequently assayed for ACCase, PCCase, MCCase and PCase activities. As shown in Fig. 7, the four carboxylase activities were not affected by increasing amounts of preimmune serum, and ACCase, PCCase and PCase activities were similarly unaffected by the introduction of the antiserum. MCCase activity, however, was gradually inhibited by the addition of increasing amounts of antiserum. Incubation of crude extracts with the anti-LB serum also specifically inhibited MCCase activity (data not shown). These results indicated that the 78 kDa biotin-containing polypeptide is the biotin-containing subunit of MCCase. The size of this subunit of MCCase from tomato is in agreement with the biotin-containing subunits of MCCases purified from animals (Lau et al., 1979), bacteria (Fall and Hector, 1977; Apitz-Castro et al., 1970) and plants (Chen et al., 1993; Alban et al., 1993).

Northern Analysis of MCCase mRNA — To determine the size of the mRNA coding for the 78 kDa subunit of MCCase, RNA isolated from the expanding leaves of tomato plants was fractionated by electrophoresis

in a formaldehyde-containing agarose gel and then transferred to a nitrocellulose filter. The blot was hybridized with the BTN cDNA (Fig. 8). A single RNA band of about 2.4 kb was detected; no additional bands were detected even upon long exposures of the blot. The size of this transcript (2.4 kb) is appropriate to code for a 78 kDa polypeptide.

MCCase Gene Organization in Tomato Genome — A tomato genomic library was screened using the TBP cDNA sequence. This resulted in the isolation of two clones, λ GT1 and λ GT12 (Fig. 9). These clones were characterized in terms of their restriction maps, and by Southern blot hybridization to position and orient the MCC-1 sequence. That these two clones had different restriction maps indicates that they represent from two different genes. In order to determine the number of MCCase genes in the tomato genome, total genomic DNA isolated from tomato leaves was digested to completion with the restriction endonucleases *HindIII*, *EcoRI* and *BglII*. The Southern blots were probed with the GKBT cDNA. A very simple banding pattern was observed, with two or three genomic restriction fragments hybridizing to the GKBT cDNA (Fig. 10). This result and the isolation of two distinct genomic clones coding for the biotin subunit of MCCase suggest that the tomato genome contains at least two MCCase genes.

DISCUSSION

We have isolated, for the first time, the genes and cDNAs coding for the biotin-containing subunit of MCCase. The cloning of MCCase represents the first biotin enzyme to be cloned from higher plants. The experimental strategy utilized to identify the biotin-containing subunit of MCCase was by using reagents generated from a cDNA clone which was initially identified as potentially coding for a biotin enzyme based on the fact that its protein product was biotinylated in *E. coli* (Hoffman et al., 1987). Thus the enzymatic function of the protein coding by this cDNA was initially unknown. The identification of the 78 kDa biotin-containing polypeptide as the biotin subunit of MCCase is only the second biotin enzyme to be identified in the plant kingdom.

MCCase from bacteria and animal kingdoms have been extensively investigated (Lau et al., 1979; Fall and Hector, 1977; Apitz-Castro et al., 1970). They have been highly purified and well characterized from *Achromobacter*, *Pseudomonas citronellis* and bovine kidney. In each case, the enzyme is composed of two different subunits. The large subunit has a molecular weight of 73,000 to 96,000 and contains biotin. The small subunit has a molecular weight of 61,000 to 78,000, and is biotin-free. Recently, MCCases have also been isolated from plants. The biotin-containing subunit of MCCase from carrot (Chen et al., 1993), pea, potato (Alban et al., 1993) and maize (Diez et al., submitted) are 78 kDa, 76 kDa, 74 kDa and 78 kDa respectively. Thus

the sizes of biotin-containing subunits of MCCases from plants are similar to the sizes of this subunits from bacteria and animals.

The amino acid sequence of the biotin subunit of MCCase, deduced from the nucleotide sequence of the cDNA clones, was compared to the NBRF protein data base. Apart from the sequence homology between MCCase and other biotin enzymes, which was discussed in the Results section, we obtained notable sequence homology between MCCase and lipoamide transferases (Ali and Guest, 1990) and carbamoyl-phosphate synthetases. The lipoic acid carrier domain of lipoamide transferases have similar sequences to the biotin carboxyl carrier domain of MCCase (33% identities over 62 residues). As pointed out previously (Lim et al., 1988), this sequence similarity maybe an indication of the homologous functions of the lipoic acid binding domains and the biotin carboxyl carrier domains, in that both cofactors act as intermediate carriers of substrates between physically separate active sites.

The carbamoyl phosphate synthetase sequences are similar to the biotin carboxylase domain of MCCase. This sequence similarity maybe an indication that the reaction catalyzed by carbamoyl phosphate synthetase may have mechanistic similarity to the biotin carboxylase reaction. Indeed, it is proposed that both reactions occur via the formation of a carboxy-phosphate intermediate, which in the case of biotin enzymes reacts with the N-1' of biotin, and in the carbamoyl phosphate synthetase reacts with ammonia.

We were initially surprised to find that one of the cDNAs coding for the biotin subunit of MCCase (GKBT) was interrupted by an intron.

However, closer inspection of the position of this intron supports the hypothesis that biotin enzymes evolved via gene fusion mechanisms (Lynen, 1979). The intron (positioned at residue #322 in Fig 2) separates the biotin carboxylase and biotin carboxyl carrier domains from each other. It has been proposed that new proteins arise in evolution by the rearrangement and sharing of exons which code for discrete structural elements, such as binding and catalytic sites (Craik et al., 1983; Padgett et al., 1986), and that the function of introns in this model is for enabling the fusion of such exons. The finding that the biotin carboxylase and biotin carboxyl carrier domains of MCCase are separated by an intron, is consistent with the hypothesis that the biotin subunit of this enzyme evolved via the fusion of exons containing each of these domains.

The initial isolation of the MCCase cDNA clone was the result of the biotinylation of its protein product in *E. coli* (Hoffman et al., 1987). The identification that this sequence codes for a biotin enzyme *in planta* establishes the universality of the structural features that are recognized for biotinylation of biotin enzymes. Thus, at least *E. coli*, is able to biotinylate examples of biotin enzymes from all phylogenetic kingdoms (Cronan, 1990). As with other biotin-containing enzymes, with the exceptions of ACCases, the site of biotinylation of the tomato MCCase is near the carboxy-terminus, where the tetrapeptide Ala-Met-Lys-Met is found. The conservation of this tetrapeptide has been postulated to be for recognition of the lysine residue that is biotinylated by the holocarboxylase synthetase (Samols et al., 1988).

However, the sequencing of the yeast (Al-Feel et al., 1992) and vertebrate (Takai et al., 1988; Lopez-Casillas et al., 1988) ACCases, and yeast urea amidolyase (Genbauffe and Cooper, 1991) established that these biotin-containing enzymes do not have this tetrapeptide; they contain Val-Met-Lys-Met and Ala-Met-Lys-Ala, respectively. In addition, substitution of leucine for either methionine in the tetrapeptide of the 1.3 S subunit of TCase from *P. shermanii* does not affect the biotinylation of the lysine residue in the tetrapeptide (Samols et al., 1988). Thus both methionines appear not to be required in the recognition for biotinylation, rather they may have a role in catalysis.

Apart from this tetrapeptide that is highly conserved among biotin enzymes, the amino acid sequence surrounding the biocytin is variable among different biotin-containing enzymes; about 25% amino acid identity over a span of about 80 amino acids (Figure 3). Indeed, MCCase from tomato shares 25% identity over an 87 amino acid overlap (MCC-1, 350-436. Figure 3) with the *E. coli* BCCP. Despite this low homology, when the cDNAs coding for plant MCCase were expressed in *E. coli*, the protein products were good substrates for the *E. coli* holocarboxylase synthetase (Hoffman et al., 1987; Figure 4 and Figure 5 in this paper). In addition, a peptide fragment containing the C-terminal 69 amino acid residues of tomato MCCase (BX3, see Fig. 5) was successfully biotinylated in *E. coli*. Thus these terminal residues contain all required information for the biotinylation of the lysine residue, in close agreement with prior findings (Cronan, 1990).

Thus the structural features that identify the lysine residue as a substrate for biotinylation by holocarboxylase synthetase are still unclear. Mutagenesis studies have shown that a hydrophobic residue 33 amino acids downstream of the lysine residue in the 1.3 S subunit of TCase from *P. shermanii* is important for either the recognition of the lysine residue for biotinylation or for the catalytic mechanism of biotinylation (Murtif and Samol, 1987), since substitution of this hydrophobic residue with glycine abolished biotinylation. That an amino acid residue, situated at a considerable distance from the site of biotinylation in the primary structure of the protein, affects biotinylation, suggests that recognition and/or biotinylation of the lysine residue maybe partially dependent on the secondary structure around the site of biotinylation.

The secondary structure surrounding the biotinylation site of biotin-containing enzymes may indeed be highly conserved (Figure 11). Secondary structure prediction indicates that the lysine residue that is biotinylated is present in the center of an α -helix in a number of biotin enzymes. Upstream of this α -helix there is little conserved secondary structure (not shown). However, downstream of the α -helix containing biocytin, there is considerable conservation in the predicted secondary structures among biotin-containing enzymes. The conserved secondary structure pattern begins four residues upstream of the biocytin, and has the features $[(\alpha\text{-helix}) - (\text{turn}) - (\alpha\text{-helix}/\beta\text{-sheet}) - (\text{turn}) - (\alpha\text{-helix}/\beta\text{-sheet})]$ (Figure 11). In addition, there are a discrete number of hydrophobic amino acids in this segment of polypeptide which are

highly conserved (Figure 11). This conserved pattern of secondary structure along with the conserved hydrophobic residues may be the structural features which identify the lysine residue for biotinylation by the holocarboxylase synthetase. Indeed, the alteration of the leucine, 33 residues down stream of the lysine residue that is biotinylated in TCase, which affects biotinylation, is also predicted to alter the last α -helix to a turn, consistent with the hypothesis outlined above. Obviously, further site specific mutagenesis studies will be required to test and modify this hypothesis.

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FIG. 1. Restriction endonuclease maps and the strategy for the determination of the nucleotide sequences of cDNAs MCC-1, BTN and GKBT. Each DNA fragment was sequenced to the extent indicated by each of the arrows, in the direction shown. Arrows beginning at open circles or closed circles indicate sequencing reactions carried with single stranded or double stranded DNA templates, respectively. Restriction enzymes marked by an asterisk are sites obtained from cloning vectors or linkers. Identical sequences in each cDNA are indicated with identical shading patterns.

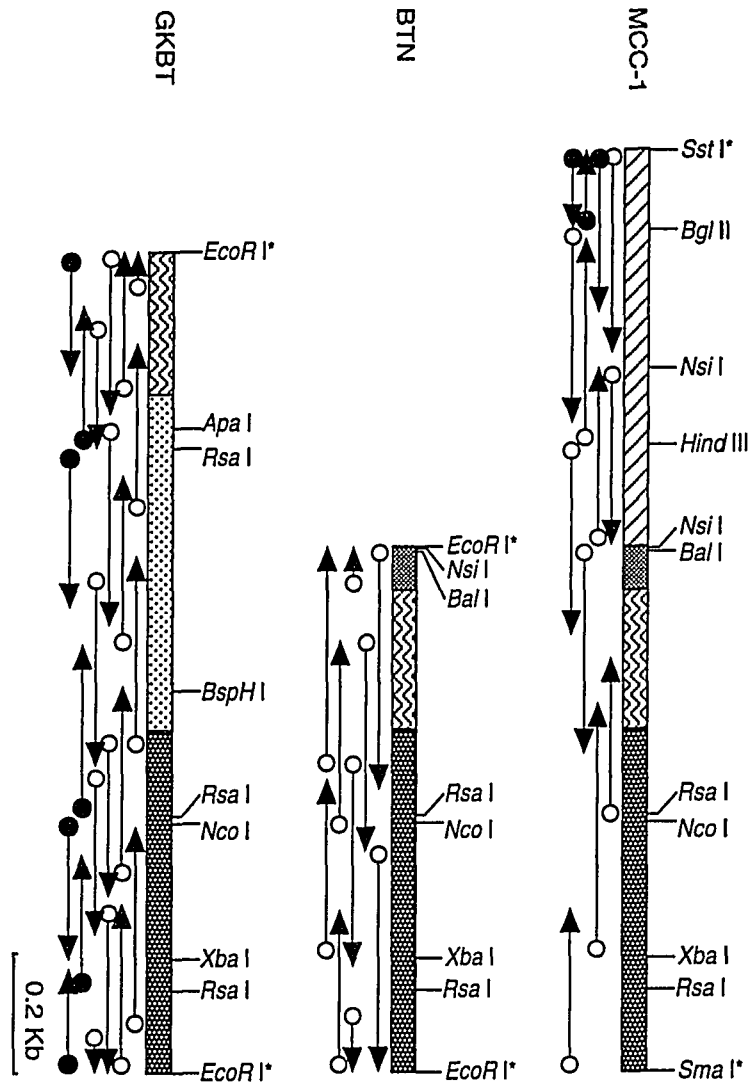


FIG. 2. Nucleotide sequence of cDNA clones MCC-1, BTN, GKBT and TBP, and the deduced amino acid sequence. The predicted amino acid sequence (single-letter code) is shown below the nucleotide sequence. Nucleotides are numbered in the 5'-3' direction beginning with the first nucleotide at the 5'-end of MCC-1. The BTN sequence begins at position 647, which is indicated. The GKBT sequence begins at position 717, which is indicated. The TBP sequence begins at position 1667, which is indicated. The intron sequence in GKBT is shown in lowercase. The cDNAs MCC-1 and BTN do not contain this intron sequence. In these sequences base #966 is juxtaposed to base #1535. The exon/intron consensus sequences are underlined and the splicing sites are marked with dots underneath the sequences. The *Rsa*I sites that delineate the GKBT sequence into four fragments, three of which were subcloned as BX1, BX2 and BX3, are indicated. The stop codon is labeled "End". The putative polyadenylation signal ACTCAAAA is boxed. The tetrapeptide Ala-Met-Lys-Met is underlined with a dashed line.

^{←MCC-1}
 1 CCACGCAATTACACAGTACGCTAGGAACCTGTTGAGTTTATAGTTGATACACAATCTGGA 60
 1 P R N Y H S T L G T V E F I V D T Q S G 20
 61 CAATTTCAATTTTATGGAGATGAATACCCGCTTCAGGTTGACGATCCAGTTACAGAGATG 120
 21 Q F H F H E H N T R L Q V D D P V T E M 40
 121 ATGTGTGTCAGATCTTCTAGAGTGGCAAATACGTTTCCAAATGGGAGCCTCTCCC 180
 41 I V G Q D L V E H Q I R V A N G E P L P 60
 181 TTGACTCAGTCAGAGGTGCCATTTTCAGGTCATGCTTTTGAAGCCCGAATTTATGCTGAA 240
 61 L T Q S E V P F S G H A F E A R I Y A E 80
 241 AATGTTCCAAAAGGATTTCTTCCAGCACTGCTGTTCTTCATCATTTATGCTGTTACA 300
 81 N V P K G F L P A T G V L H H Y C P V T 100
 301 GCTACATCAGCAGTTAGAGTTGAGACTGGAGTGGAGGAAGGGGACACAGTAAGCATGCAT 360
 101 A T S A V R V E T G V E E G D T V S M H 120
 361 TATGATCCCATGATTGCAAGCTAGTAGTATGGGACAGGACCGCCTTTCAGCAGTATA 420
 21 Y D P M I A K L V V W G Q D R L S A L I 140
 421 AAAATGAAGGATTGCTGTCAAAGTTTCAGGTTGCAAGTTTACCCCAATATAGACTTT 480
 141 K H K D C L S K F Q V A G L P T H I D F 160
 481 ATCATTAAGCTTGCCAGTCATAGGCTTTTCAAATGGTGAAGTAGAACTCATTTTATT 540
 161 I I K L A S H R A F Q N G E V E T H F I 180
 541 GAGCGGTACAAGGATGATCTGTTCTATAGATGGTTCCAATCCAATATCAGCAGAAAAGCT 600
 181 E R Y K D D L F I D G S H P I S A E K A 200
 601 GAGAGTGCTGCCAAACCATCTAGCTGCATCCATTTGAGCTGCATGCATATTTGGCCAAAT 660
 201 E S A A K P S S C I H C S C H H I G Q H 220
 661 GAATTCGAAGATTGAAGGATAAGGCTCTGGAGGCTTCATTTGTGTTATGTTATCTCT 720
 221 E L A R L K D K A P G G L H L W Y G N P 240
 721 CCATTCAGAATCAATCATTTTCTTAAGCGAAGCAGTGATCTTGAATGGGAGAATCAATAC 780
 241 P F R I N H F A K R T V D L E W E N Q Y 260
 781 AGTATTAGTGGTTCAAATCTTTTAAAGCTCTCCATCACCTATTTCAGATGGGAAATAC 840
 261 S I S G S N L L T V S I T Y L P D G K Y 280
 841 CTAGTTGAGACAGGAGAAAGCAATCCCTGGTTTGGAGATCCAGGTGACACAATAGC 900
 281 L V E T G E S N S P G L E I Q V T Q L S 300
 901 AACAACTACTATAGAGTTGAAGTGGATGGTATAGCCTGAATGTCTGTTTGGCAGCTTAC 960
 301 N N D Y R V E V D G H S L N V C L A A Y 320

961 TCCAAGGtaacaccttatatttcaggctcttatcttcaagctgtcctgggccccctggcat 1020
 321 S K 322
 1021 acatattgactgacaagtacacacactatgttttaggctgaggctggagttattaaatgtc 1080
^{Res 1}
^{EX1} ^{EX2}
 1081 taacattgcacaacagtgatgaattatagcactaaattttgtatattaggtgttttt 1140
 1141 aaggttgtgaattgtgcacttcacttcaaagaagatgcacttgctgttaccttttgct 1200
 1201 tcaggccctatggacttttttctgtgcacattttgtctattaagaacactatcttttaa 1260
 1261 caccattttcaatctagtcggcctatgaaaaagaaataaaagagagaagattaacct 1320
 1321 tctagtgcctcgtgtgtaaaattttccagggtggtgagaatggtgcaacttagtcca 1380
 1381 ctgaatacaatttggaaactaaagatcaagggtttgtcatatcttttagcacatttagcttt 1440
 1441 gttcatgagaatctgatgcccttgatcaagaatgcagtgattgccaaggaaaaccgat 1500
 1501 ccaaatattagtttgccaaacttccattttttgcagGATCAAAATGAGCATATTCACATCTG 1560
 323 D Q I E H I H I W 331
 1561 GCATGGGACTCTCAACATCAGTTTAAACAAGGATGGGCTAGAAATCTATGATGATGA 1620
 332 H G D S Q H Q F K Q R M G L E I Y D D E 351
 1621 AGAAACAATAGACAAGCCTGTAGGGTGGGACATCTTATCTCTGTGACTGTGATTGC 1680
 352 E T I D K P A R V A T S Y P S ^{EX2} ^{Res 1} ^{EX3} ^{TBP} T V I A 371
 1681 ACCCATGGCTGGGCTAGTGGTTAAAGTATTTGGTGAAGGATGGGAGAAAGTTTCAAGAGG 1740
 372 P H A G L V V K V L V K D G E K V Q E G 391
 1741 ACAACCTGTGTAGTATTAGAAGCAATGAAGATGGAGCATGTAGTAAAGCACCAGCTAA 1800
 392 Q P V L V L E A H K H E H V V K A P A N 411
 1801 TGGCTATGTAAAGGGCTTGAATCAAAGTGGGCCAATCGGTCCAAGATGTTATAAACT 1860
 412 G Y V S G L E I K V G Q S V Q D G I K L 431
 1861 CTTTGCTCTCAAGGACTGAAATATATCTCGAGCTATGACAACATCATTTAGACAACTG 1920
 432 F A L K D End 436
^{EX1} ^{Res 1}
 1921 TATGTATGACCTTCCTATGAGGACTGTACATCCACTAGGCATAAGAATAACAACATTGAG 1980
 1981 ATGTAAAATCTCTTGAGTTTCATCTTACATTTCAAATACATTTACTTGTAATGACT 2040
 2041 TTCCA[ACTCAAA]TTTGTCCAGATTAGTGATATATGATCAATGGTGTCTCATTA(n) 2095

FIG. 3. Comparison of the amino acid sequence deduced from the nucleotide sequence of MCC-1 with sequences of other biotin-containing enzymes. This comparison was generated by the BESTFIT program of the GCG software package. Residues identical to the predicted amino acid sequence of MCC-1 are boxed. Full stops indicate gaps inserted to optimize the alignment. The filled circle above the lysine residue at position 401 of the MCC-1 sequence represents the biotinylation site. Asterisk indicates the carboxy terminus of the protein. The biotin carboxylase domain and the biotin carboxyl carrier domain are labeled at the right side of the sequences. The filled triangle at position 322 of the MCC-1 sequence indicates the position of the intron in the GKBT cDNA. The abbreviations used are: MCC-1, the deduced amino acid sequence from the tomato cDNA clone MCC-1; Ch-Acc, chicken acetyl-CoA carboxylase (Takai et al., 1988); Hu-Pcc, α subunit of human propionyl-CoA carboxylase (Lamhonwah et al., 1989); Y-Pc, yeast pyruvate carboxylase (Lim et al., 1988); E-Bc, biotin carboxylase component of the *E. coli* ACCase (Kondo et al., 1991; Li and Cronan, 1992); E-Bccp; biotin carboxyl carrier protein of *E. coli* ACCase (Rylatt et al., 1977); P-Tc, 1.3 S subunit of methylmalonyl-CoA:pyruvate transcarboxylase from *P. shermanii* (Murtif et al., 1985). In each of these sequences residues are numbered as in the cited reference.

MCC-1	CAVRLAKMVGYSIA.GTVRELYL.SQDSSTHLELN.PRLQVDPFVTEML.	441
Ch-Acc	AVALLARAVYSIA.GTVRELYL.SQDSSTHLELN.PRLQVDPFVTEML.	452
Hu-Pcc	DAVKLAKCCYRNA.GTNEPLVEMQNH.HFTLHN.PRLQVDPFVTEML.	340
E-Bc	CA.KACVDIDYRGA.GTNEPLFE..NGSEYEDENNVTTHLOVSEHYTEML.	323
		300
MCC-1	..VGQDLVEMQINVANGSEPL.....F...LQSEYTHES..	69
Ch-Acc	..ADONLEPANOLOLMGTLPHIRKID.IRMVGVSH...WODGSTIDENS	495
Hu-Pcc	WGPSPGKVTLOEHTSGVNLIF.....EQLQHPQDKITTR..	366
E-Bc	..TQIDHMAQDQIMAGASU.....SINGPEV....	354
	..TQVDLKEQTEHNGQEL.....SINGPEV....	328
MCC-1GRHFEARITYAENVKRG.F.LPATGVLHHNCPVTATSAVRENLG	110
Ch-Acc	AHVCPRHGYINARITISEN.FEDGFI.HSSSGTYQEZL.NFRSRKNVGVYPS	542
Hu-PccGNAVGEGRVYASD.PYKSPQLSSJGQEPHLHPGVAYDSG	408
E-BcGEALIQCRITVSD.PAKNP.OPIHTGRILEVY.RSAGNGVRLDQSHV...FGHAMEGRINLASED.FNT.F.LHSPFGKITRFRHA.PGFGQVYMWESH	394
		370
MCC-1	RESQELV.SAHVYDPMNLKILVWKQDRL.SALTEKQCLSNBOVAK.L.FPTNL	158
Ch-Acc	VAAAGG.LIEFPAISQEGHCF.SQWDEINREHAF.SKNTVNLKELSTRQDPRETV	591
Hu-Pcc	IQHGSQ.LSLIYDPMISKHITVQSDIDYETALIKNAALNDNYIRQ.VTHNL	456
E-Bc	NAYAGTILSPHYSHALVWCSGCSSTVEIVREMLIRALIEHRIQVATNIP	444
	ITYAGTVLWPPYVSHNGLKILCYKGENRDVLIARLNALQELTIIDG.IWTVN	418
MCC-1	EFITKLAISHRAFQNGSEVTHNLENYHNDLPIDGSNPISAEVNL.SNLRKPS	207
Ch-Acc	EYLKILLETESFGONRITLHGLNLIAE.....RVQASRPD.TNL...	631
Hu-Pcc	ALIREVILINSREYVKGDISIKMEL...SEVYHEDPKHMLTMSQ.KNQL...	499
E-Bc	FILTELETPNVFIEGTGYNQFIDIDPFGQWVSSORAKLHVLADVDNG	495
	DLQIRIANDENEGHGNNHLYIEK.FLGLQZK*	449
MCC-1	SCIRQSC.MHJGONELARLKDKRPGGHLNWTGKPPRI..NHFKFTVTL	254
Ch-Acc	.GVVQGA.LHVLAD...VSFRNSVSNFLHSLERGQVLP.A.HTLIN.LTVYV	673
Hu-PccLALASSLFVAFQNLH...QHFOENSRMVPVIRKPIDINMELSY	537
MCC-1	PMENO.YQLSGESLUTLQITVLPDQRYVLEIDESNSPGLDQVY.CLSN	301
Ch-Acc	EL.....LYEGGRVYVAVKVRKOSPNSVYVLE.N.NSCEVLEVYV.NLSD	712
Hu-Pcc	KLHDKHVVYVANS.....NGSVFSLV.VDQSK...LNVTSYVNLAS	574
E-Bc	PRVYEDFOKMRREYGDIDSVLPYRSPSPILHDPB....LEIPY.....	1040
	MDIRKIRKIDELVEEGISIELISDGE.ESEV	30
MCC-1	NDYEVEDVQSLNVCULVYSPDOIEHNTMNGSGLQFKQKMGLEIYDHP	351
Ch-Acc	GGLLLSYDQSSYVYVY...EEVDYVRYHTIGKTC.....VFEVLE	748
Hu-Pcc	PILSVSYDQTRTQOCLSRBAGNMSEIQPFLGTGVYVNLITLAAEL...NK	622
E-BcVIEOGKTHLIRKLOAVGDINKKTGEREVYFDLNGEMRKIRIVAS	1082
	RISFNA.PAASFVVMQALYAAPMOOPAOASNAAPATVPSEAPAAAZIS	79
	MTLKVTVNGTAYDVVDVDEKSEINPMTITLPGGGTGC	36
MCC-1	ETLLEPFRVADS...EPSGTVLIA.PNAGLVTVLVVDQDEKYEGQEPVLYE	398
Ch-Acc	ND.....ES.LIRSHSAKLIQYVVEDGHHFAGCCFAPATIE	784
Hu-Pcc	PTLEH.....VTEEDISSVTRSPRHHGVYVANSVRGDNVMEGDEICVLE	665
E-Bc	RSQRTVEVTKSRADMDPLHIGAPMAGVTVMEVNRHNGSLIKRGOBNAVLS	1132
	GHLYRSEWYVCP...LHTPSPIH.....NAPEVYCKVYVNDTICVIB	119
	GAPAPRPAAGGAGAKRAGEEILHLELAGTVLSNLEVNESDTPKNGTVLVYIE	86
MCC-1	ANKHEHVYVABANKVLSGLEHMGSGVQDGLKLPANLKH*	436
Ch-Acc	VMKRVMTLTLGSSGCIHYV.RHGAVLDBGVIALQLODPSRVOQAEHLH	833
Hu-Pcc	ANKQNSMTLNGKTCIKRSYHONCDNPGDEBDILVILE*	702
E-Bc	ANKHEKITSFSDIQKREVFSLDENVDSDLVYLEQOVPEVYKA*	1178
	ANKKENGIEHDKSGVTKALIVEGGHVEFDEPVLVIE*	156
	ANKHEPEINAPVDGVEKVLVERDNYVQGGCQILKIG*	123

biotin carboxylase domain

biotin carboxyl
carrier domain

FIG. 4. Expression of the BTN cDNA in *E. coli* as a glutathione S-transferase fusion protein. The partial cDNA clone BTN was fused, in frame, to the 3'-end of the glutathione S-transferase gene in the plasmid pGEX-2T (Pharmacia). Panel A is a schematic of the resulting GEX-BTN chimeric gene. The nucleotide and predicted amino acid sequences at the junction between the glutathione S-transferase gene and the BTN cDNA are boxed. P_{tac} is the IPTG inducible *E. coli* hybrid *tac* promoter. The expression vector, pGEXBTN, was introduced into *E. coli* and expression was induced with IPTG. Aliquots of protein extracts were fractionated by SDS-PAGE in duplicate gels. One gel was stained with Coomassie Brilliant Blue (panel B), and the other was subjected to Western blot analysis with ^{125}I -streptavidin to identify biotin-containing polypeptides (panel C).

Proteins were extracted from the host *E. coli* strain, JM105, harboring no plasmid (lane 1), harboring the parent plasmid pGEX-2T (lane 2) and harboring the recombinant plasmid pGEXBTN (lane 3).

The expressed glutathione S-transferase-BTN chimeric protein (57kDa) was purified by affinity chromatography with immobilized avidin. The purified protein was analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue (panel D).

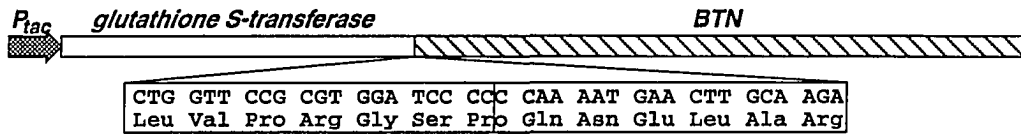
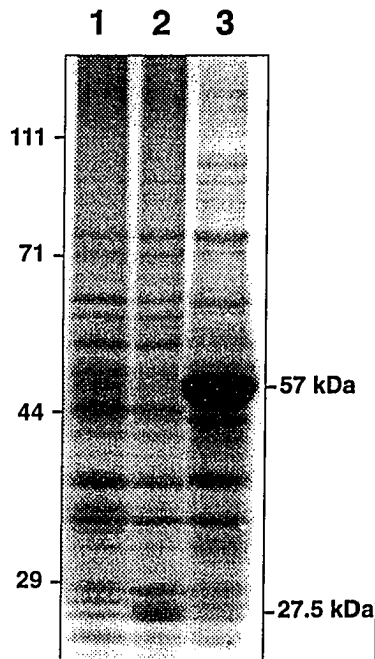
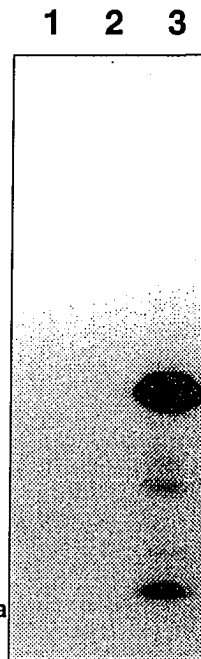
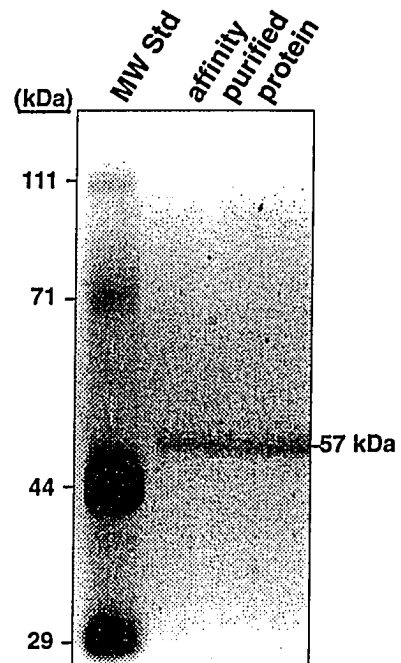
A**B****C****D**

FIG. 5. Expression of the 3'-end of the MCC-1 cDNA in *E. coli* as β -galactosidase fusion protein. An *Rsa*I-*Xba*I DNA fragment, from the 3'-end of the MCC-1 cDNA (nucleotide 1670 - 1914 in Figure 2) was fused, inframe to the 3'-end of the β -galactosidase gene in the plasmid pUR289. Panel A is a schematic of the final construct of the β -galactosidase-BX3 chimeric gene, we called LB. The nucleotide and predicted amino acid sequences at the junction between the β -galactosidase gene and BX3 is boxed. *P*_{lac} is the *E. coli* lac promoter. The resulting expression vector pLB was introduced into *E. coli* and expression was induced with IPTG. Aliquots of protein extracts were fractionated in triplicate by SDS-PAGE. The resulting gels were either stained with Coomassie Brilliant Blue (panel B), or subjected to Western blot analyses. One blot was sequentially incubated with mouse anti- β -galactosidase, antimouse IgG and ¹²⁵I-Protein A to identify β -galactosidase and β -galactosidase fusion proteins (panel C). The other blot was incubated with ¹²⁵I-streptavidin to identify biotin-containing polypeptides (panel D).

Proteins were extracted from the host *E. coli* strain, JM105, harboring no plasmid (lane 1), harboring the parent plasmid pUR289 (lane 2) and harboring the recombinant plasmid pLB (lane 3).

The expressed β -galactosidase-BX3 chimeric protein was purified by affinity chromatography with immobilized avidin. The purified protein was analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue (panel E).

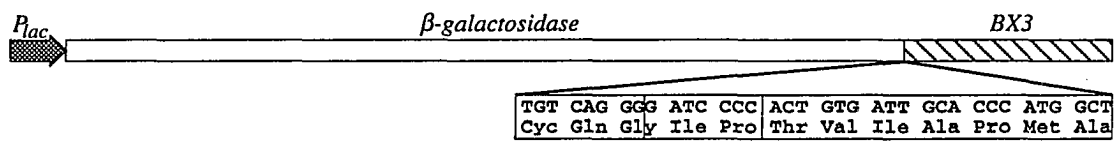
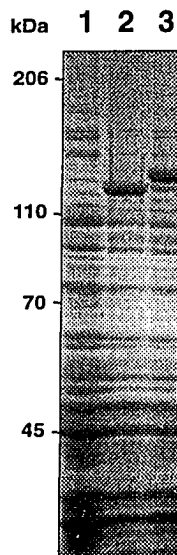
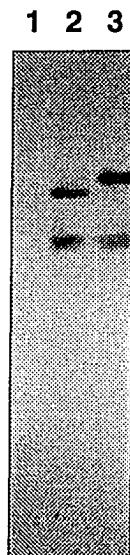
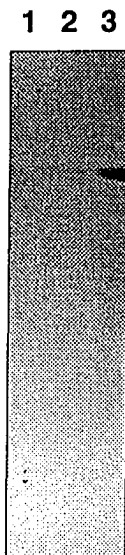
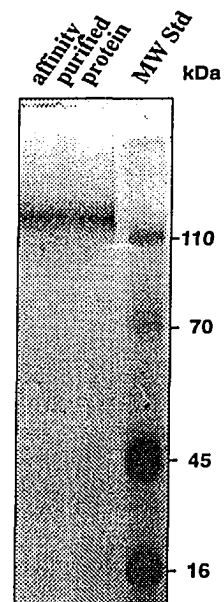
A**B****C****D****E**

FIG. 6. Identification of the protein coded by the MCC-1 cDNA. A denatured protein extract (lane 1) from tomato leaves was subjected to affinity chromatography with immobilized avidin (lane 2) or with immobilized avidin pretreated with 2 mM biotin (lane 3). After extensive washing of the columns, biotin-containing polypeptides were eluted with boiling 2% SDS and collected. These three fractions were subjected to SDS-PAGE and Western blot analysis. The blot was sequentially incubated with anti-GEX-BTN serum and ^{125}I -Protein A.

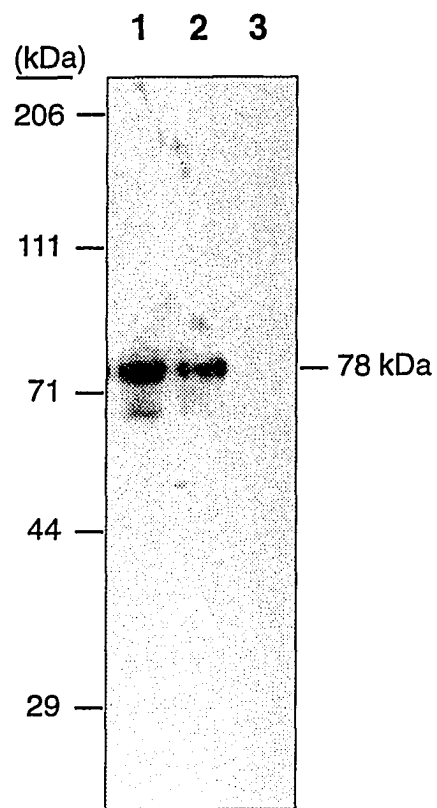


FIG. 7. The MCC-1 cDNA codes for the biotin-containing subunit of MCCase. Increasing amounts of preimmune serum or anti-GEX-BTN serum was added to aliquots of an extract from tomato leaves. Following an incubation on ice for 40 min, residual activities of MCCase, ACCase, PCCase and PCase were determined.

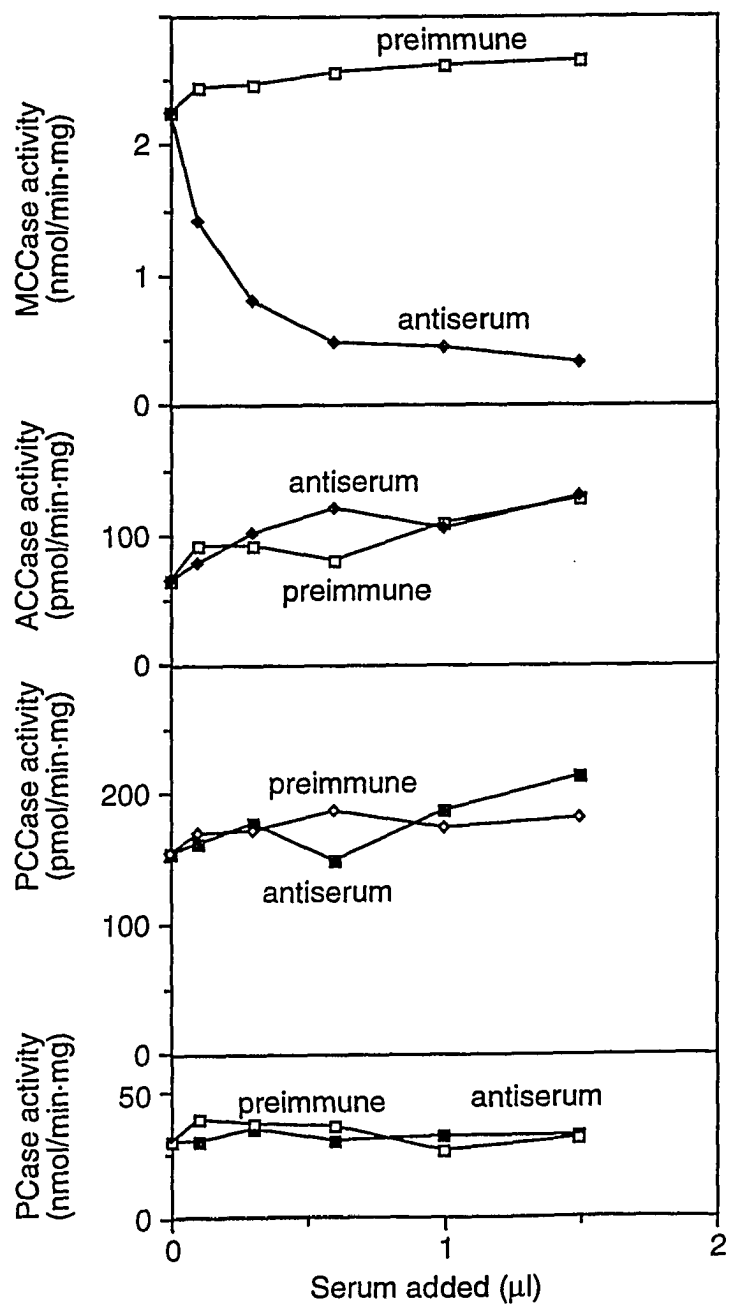


FIG. 8. Identification of the mRNA coding for the biotin-containing subunit of MCCase. RNA was isolated from expanding leaves of tomato plants and fractionated by electrophoresis in a formaldehyde-containing agarose gel. The resulting gel was stained with EtBr (panel A), and transferred to a nylon membrane. The mRNA coding for the biotin-containing subunit of MCCase was detected by hybridization with ³²P-labeled BTN cDNA (panel B). The sizes of the RNA species used for calibration are labeled on the left side.

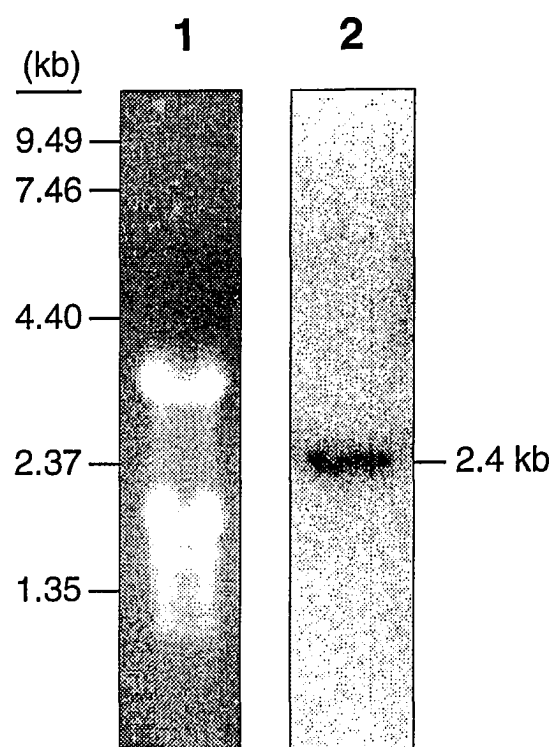
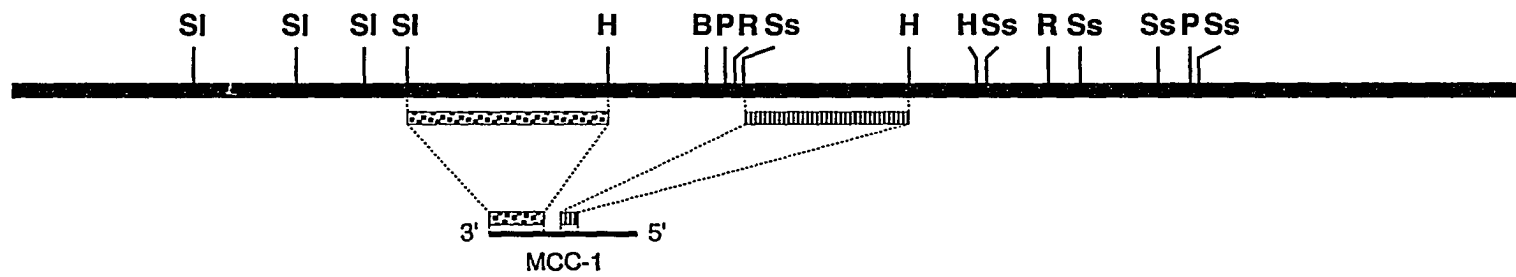


FIG. 9. Restriction maps of isolated tomato MCCase genes.

Two recombinant bacteriophage (TG1 and TG12) were isolated from a tomato genomic library by hybridization with the TBP cDNA. The thick horizontal lines represent cloned tomato genomic DNA. The thin horizontal line represents the MCC-1 cDNA. The orientation of the MCC-1 sequence is labeled. The positions of restriction sites within the genomic DNA are indicated. Cross-hybridization between 5'-end-specific and 3'-end-specific probes from the MCC-1 cDNA and fragments from TG1 and TG12 are indicated by similarly shaded boxes. Restriction sites for *Bam*HI (B), *Eco*RI (R), *Hind*III (H), *Pst*I (P), *Sal*I (S), *Sst*I (Ss) and *Xba*I (X) are indicated. TG1 has no *Xba*I and *Sma*I restriction sites and TG12 has no *Bam*HI restriction site.

TG1



TG12

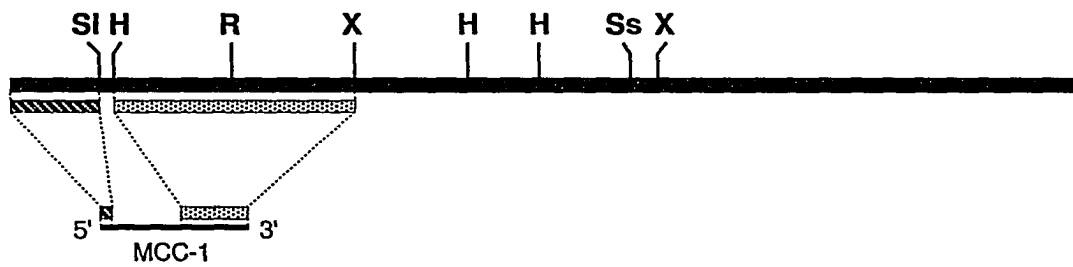
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FIG. 10. Identification of genes coding for the biotin subunit of MCCase by Southern blot analysis of tomato genomic DNA. Tomato genomic DNA (10 μ g) was digested with the restriction endonuclease *Hind*III, *Eco*RI or *Bgl*II, and fractionated by electrophoresis in an agarose gel. Following transfer of the DNA to a nylon membrane, the gene sequences coding for the biotin-containing subunit of MCCase were detected by hybridization with 32 P-labeled GKBT cDNA.

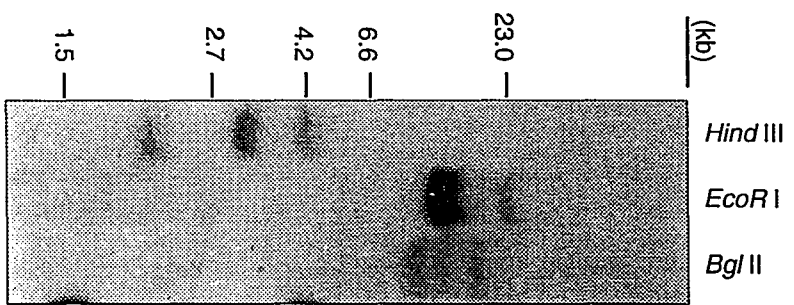


FIG. 11. Comparison of the predicted secondary structures surrounding the biotinylation sites of biotin enzymes. Protein secondary structures were predicted by the PEPTIDESTRUCTURE program of the GCG software package. The predicted secondary structures are indicated at the top of the sequences. The sequences predicted to be in α -helix structure are underlined with a solid line. The sequences predicted to be in β -sheet structure are underlined with a dashed line. The sequences predicted to be in a turn are underlined with a dotted line. The conserved hydrophobic amino acid residues are marked with dots at the bottom of the sequences. "MK", labeled at the bottom, is the conserved dipeptide found in all biotin enzymes, where the lysine residue is the biotinylation site. The abbreviations used are as the same as in Figure 3.

Secondary structure	<u>α-helix</u>	<u>turn</u>	<u>α-helix/ β-sheet</u>	<u>turn</u>	<u>α-helix/ β-sheet</u>
MCC-1	<u>LEAMKMEHVVKA</u>	<u>PANG</u>	<u>YVSGLEIKVGQSV</u>	<u>QDG</u>	<u>IKLFALKD</u>
Ch-acc	<u>IEVMKMVMTLTA</u>	<u>GESG</u>	<u>CIHYVKRPGAVLD</u>	<u>PG</u>	<u>CVIAKLQL</u>
H-pcc	<u>IEAMKMNSMTA</u>	<u>GKTG</u>	<u>TVKSVHCOAGDTVG</u>	<u>EG</u>	<u>DLLELE</u>
Y-pc	<u>LSAMKMEMIIS</u>	<u>PSDG</u>	<u>QVKEVFVSDGEN</u>	<u>VDSSD</u>	<u>LLVLLED</u>
E-bccp	<u>VEAMKMMNQIEA</u>	<u>DKSG</u>	<u>TVKAILVE</u>	<u>SG</u>	<u>QPVEFDE</u>
P-tc	<u>LEAMKMETEINA</u>	<u>PTDG</u>	<u>KVEKVLVKERDAVQ</u>	<u>GG</u>	<u>QGLIKIG</u>
Consensus sequence	● ●MK● ●	G	● ●	G	●

**PAPER II. STRUCTURAL AND IMMUNOLOGICAL
CHARACTERIZATION OF β -METHYLCROTONYL-CoA
CARBOXYLASE FROM TOMATO**

**STRUCTURAL AND IMMUNOLOGICAL CHARACTERIZATION OF β -
METHYLCROTONYL-CoA CARBOXYLASE FROM TOMATO**

Xun Wang¹, Eve Syrkin Wurtele² and Basil J. Nikolau^{1†}

¹Department of Biochemistry and Biophysics, and ²Department of Botany, Iowa State University, Ames, Iowa 50011

Corresponding Author: Dr. Basil J. Nikolau

Department of Biochemistry & Biophysics,
Iowa State University,
Ames, IA 50011
Phone: (515)294-9423
FAX: (515)294-0453

Running Title: β -methylcrotonyl-CoA carboxylase from tomato

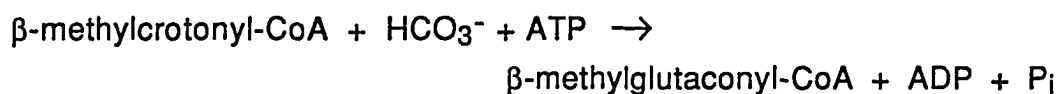
ABSTRACT

β -Methylcrotonyl-CoA carboxylase is a biotin-containing enzyme that catalyzes the ATP-dependent carboxylation of β -methylcrotonyl-CoA. The enzyme was purified 200-fold from tomato leaves. The native enzyme has a molecular weight in the range of 1,000,000 to 1,400,000, as measured by gel filtration chromatography and two non-denaturing polyacrylamide gel electrophoresis methods. The enzyme is composed of two types of subunits, a biotin containing subunit ($M_r = 78,000$) and a biotin-free subunit ($M_r = 60,000$). The two subunits occur at equal molar ratio in the enzyme. Antisera prepared against the biotin subunit of this enzyme of tomato cross-reacts with this enzyme from soybean and maize, but not with the bovine enzyme. Two domain-specific antisera were generated for the biotin subunit of MCCase. One of these antisera, anti-BX3 serum, recognized epitopes on the biotin carboxyl carrier domain. The other antiserum, anti-BX1 serum, recognized epitopes that occur between the biotin carboxyl carrier domain and the biotin carboxylase domain, a region of the protein that shows low sequence similarity among all biotin enzymes. Whereas anti-BX3 serum could inhibit the activity of the enzyme, anti-BX1 serum was unable to inhibit the enzyme. We interpret these data to suggest that the region of the polypeptide between the biotin carboxyl carrier domain and biotin carboxylase domain, which is minimally

conserved among biotin enzyme sequences, does not directly contribute the structure(s) necessary for catalysis.

INTRODUCTION

β -Methylcrotonyl-CoA carboxylase (MCCase; 6.4.1.4) is a biotin-containing enzyme that catalyzes the ATP-dependent carboxylation of β -methylcrotonyl-CoA (MC-CoA) to form β -methylglutaconyl-CoA (MG-CoA):



The reaction catalyzed by MCCase is required for leucine degradation (review: Moss and Lane, 1971) and is possibly involved in the "mevalonate shunt" (Popjak, 1971). Deficiency of this enzyme in human results in the inherited disease, β -methylcrotonylglycinuria (Stokke et al., 1972; Weyler et al., 1977). MCCases from bacteria and animal kingdoms have been highly purified and well characterized from *Achromobacter*, *Pseudomonas citronellis* and bovine kidney (Schiele and Lynen, 1981; Fall, 1981; Lau and Fall, 1981). In each case, the enzyme is composed of two different subunits. The large subunit has a molecular weight between 73,000 and contains the biotin prosthetic group. The small subunit has a molecular weight between 61,000 and 78,000 and is biotin free. The molecular weight of the holoenzymes is between 520,000 and 835,000.

MCCase was only recently identified in the plant kingdom (Wurtele and Nikolau; 1990), and characterization are now being undertaken (Alban et al., 1993; Yang et al., 1993; Diez et al., Wang et al., Song et

al., submitted for publication). Here we report the isolation of MCCase from tomato and the characterization of these enzymes in terms of their structure and catalytic functions.

MATERIALS AND METHODS

Materials — β -Methylcrotonyl-CoA, d-biotin, E-64, PMSF, HEPES, Agarose-Cibacron Blue, streptavidin, bovine kidney and liver acetone powders were purchased from Sigma Chemical Co. Other chemicals were purchased from Fisher Scientific Inc. The radioisotopes were purchased from Amersham. Avidin-agarose were purchased from Pierce. Q-Sepharose was purchased from Pharmacia. Bio-Gel P-6DG was purchased from Bio-Rad Laboratories. Protein standards for molecular weight determinations by SDS-PAGE were obtained from BRL. Native protein standards for molecular weight estimation were purchased from Sigma. Nitrocellulose was purchased from MSI. Polyethylene glycol (PEG) 8000 was purchased from Unites State Biochemical.

Tomato (*Lycopersicum esculantus* var. *Rutgers*) were grown in a greenhouse at 18-22 °C under supplemented illumination conditions with a 15h day length. Leaves were harvested 60-80 days after planting. Maize was grown in sand in the greenhouse at 25-30 °C but otherwise under the same conditions as for tomato plants. Leaves were harvested two weeks after planting.

Assay of MCCase — MCCase activity was measured as described, which was based on the MC-CoA dependent incorporation of radioactivity from $\text{NaH}^{14}\text{CO}_3$ into the acid-stable product, MG-CoA (Wurtele and Nikolau, 1990).

Production of Antisera — The production of antisera against the biotin-containing subunit of MCCase from tomato has described elsewhere (Wang et al., submitted). In brief, cDNAs coding for the different portions of the biotin-containing subunit of MCCase from tomato were expressed in *E. coli* as chimeric proteins. The expressed proteins were purified and used as antigens for immunization of rabbits.

Protein Analysis — SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described (Laemmli et al., 1970).

Proteins were transferred from the gel onto nitrocellulose membranes with a PolyBlot transfer apparatus (American Bionetics, INC.), at 450 mA for 30 min. Immunological detection of proteins was with an appropriate dilution of antisera and ^{125}I -protein A. Biotin-containing polypeptides were detected with ^{125}I -streptavidin (Nikolau et al., 1985).

Protein concentration was determined by the Bradford method (Bradford, 1976). BSA was used as a standard.

The native molecular weight of MCCase from tomato leaves was estimated by electrophoresis and gel permeation chromatography. The MCCase used for molecular weight estimation was the MCCase containing fractions obtained from Q-Sepharose ion-exchange chromatography.

The molecular weight of tomato MCCase was estimated by gel permeation chromatography as described (Laurent and Killander, 1963) with a Sephacryl S-400 gel filtration column (1.5 X 100 cm).

Two gel electrophoretic procedures were applied for molecular weight estimation. One is the exclusion limits by 2-16% linear gradient non-denaturing polyacrylamide gel electrophoresis described by Hedrick and Smith (1968). The other is a method developed by Lambin and Fine (1979) based on the mobility of the enzyme on 2-16% linear gradient non-denaturing polyacrylamide gel electrophoresis. After electrophoresis, protein standards were visualized by Coomassie Brilliant Blue staining and MCCase was detected by Western analysis probed with either the anti-MCCase serum and ^{125}I -protein A or ^{125}I -streptavidin, or both.

Preparation of Cell-Free Extracts — Plant material was frozen in liquid nitrogen, and while frozen pulverized with a mortar and pestle. The resulting powder was further homogenized after addition of two volumes of pre-chilled enzyme extraction buffer (100 mM HEPES-KOH, pH 7.0, 1 mM EDTA, 20 mM 2-mercaptoethanol, 100 $\mu\text{g/ml}$ PMSF, 1 mM E-64, 20% (v/v) glycerol, and 0.1% (v/v) Triton X-100). After the extract thawed, it was filtered through four layers of cheesecloth and the filtrate was centrifuged at 12,000g for 10 min. Low molecular weight molecules were removed from the supernatant by passage through a Bio-Gel P-6DG gel filtration column.

To prepare protein samples for SDS-PAGE, 0.5-1.0 g of tissue was cut into small pieces and extracted with 1-2 ml of the same enzyme extraction buffer as indicated above in a pre-chilled (at 4 °C) 5 ml glass tissue grinder.

Purification of MCCase — MCCase from tomato leaves was purified by a three-step procedure carried out at 4 °C. PEG 8000 powder was slowly added to crude cell-free extract, made from 1 kg of tomato leaves, to a final concentration of 16% (w/v). After all the PEG 8000 was dissolved, the mixture was centrifuged at 10,000*g* for 30 min. The pellet was resuspended in 400 ml buffer A (20 mM HEPES, pH 7.0, 1 mM EDTA, 20 mM 2-mercaptoethanol, and 20% (v/v) glycerol). The resulting solution was clarified by centrifugation at 10,000*g* for 10 min. The supernatant was passed through a Q-Sepharose column (2.0 X 20 cm) equilibrated with buffer A. This ion exchange column was washed with 2 liters of buffer A, and the bound proteins were eluted with a 400 ml 0-0.7 M KCl linear gradient buffer A. The fractions containing MCCase activity were pooled and passed through a 5 ml avidin-agarose column. The column was washed with 250 ml of buffer A. To elute MCCase, the column was equilibrated with 5 ml of 100 mM Tris-HCl, pH 7.0, 1 mM EDTA, 2% SDS and incubated in a bath of boiling water at 100 °C for 15 min. The eluate was collected by centrifuging the column at 4,000*g* for 10 min and collecting the flow through buffer.

RESULTS

Purification of MCCase — We have recently identified a previously isolated cDNA clone of tomato (Hoffman et al., 1987) as coding for the biotin subunit of MCCase, a biotin-containing polypeptide of 78 kDa (Wang et al., submitted). Utilizing streptavidin to detect MCCase via its biotin prosthetic group or antibodies directed against portions of the biotin subunit, we undertook the studies described here in to investigate the structure and enzymatic function of the tomato MCCase.

To carry out these studies, MCCase was partially purified from extracts of tomato leaves by a two-step procedure consisting of precipitation with PEG and anion exchange chromatography on Q-Sepharose. This procedure purified MCCase 200-fold to a specific activity of 850 nmol/min·mg protein, with nearly complete recovery of MCCase activity. Most importantly this procedure purified MCCase free of other biotin enzyme activities; no ACCase, PCCase or PCCase activities could be detected in the MCCase preparation. In addition, Western blot analysis of the MCCase preparation with ¹²⁵I-streptavidin or anti-MCCase serum indicated that the biotin subunit of MCCase was the only biotin enzyme present in this preparation (Fig. 1).

Subunit Composition — Silver staining of SDS-PAGE analysis of the partially purified MCCase preparation from tomato indicated a few polypeptides were present in the fractions obtained from the Q-Sepharose column that contained MCCase. One to these, of 78 kDa,

corresponded to the polypeptide detected by Western analysis of the preparation with ^{125}I -streptavidin. This polypeptide represents the biotin subunit of tomato MCCase. A second prominent polypeptide of about 60 kDa, coeluted from the Q-Sepharose column with MCCase activity and the 78 kDa biotin subunit of MCCase. Because of this behavior of the 60 kDa polypeptide we suspected that it was a second subunit of the tomato MCCase.

To test this hypothesis we passed the MCCase preparation over an avidin-agarose matrix. The material that flowed through the column did not contain MCCase activity, thus, as expected all the MCCase enzyme present in the preparation was bound to the immobilized avidin. Following extensive washing of the matrix, to remove nonspecifically bound proteins, biotin-containing proteins were eluted with boiling 2 % SDS. The two fractions obtained from chromatography on avidin-agarose, the avidin nonbinding and the avidin-binding fractions, along with an aliquot of the original MCCase preparation were analyzed by SDS-PAGE following by either silver staining (Fig. 2A) or Western analysis with ^{125}I -streptavidin (Fig. 2B). In a parallel control experiment, the identical procedure was undertaken, however, the avidin-agarose matrix was first preincubated with 2 mM biotin to block all biotin binding sites on the matrix.

These experiments confirmed that the 78 kDa polypeptide present in the MCCase preparation was indeed a biotin-containing polypeptide, since it was retained by the immobilized avidin and could only be eluted with boiling SDS. In addition, this polypeptide was not retained

by the matrix that had been pretreated with biotin. The 60 kDa polypeptide which we suspected was a second subunit of MCCase behaved in a similar manner, even though it did not contain biotin. That is the polypeptide was retained by the immobilized avidin and was only eluted with boiling SDS, and pre-treatment of the avidin matrix with biotin eliminated the ability of the matrix to retain the 60 kDa polypeptide. We surmise that the 60 kDa polypeptide is bound to the immobilized avidin via an interaction with the 78 kDa biotin-containing polypeptide, and we therefore conclude that these two polypeptides are in a complex, the MCCase holoenzyme.

Densitometric scans of the silver stained gels of the avidin-binding fraction indicated that the 78 kDa biotin-containing polypeptide and that 60 kDa nonbiotin-containing polypeptide are in an equal molar ratio. Therefore, the tomato MCCase holoenzyme is composed of equal number of biotin-containing and non-biotin-containing subunits.

Molecular Weight of the Native MCCase — To estimate the molecular weight of the native MCCase enzyme from tomato, three different procedures were employed. In all three instances, the MCCase preparation obtained from the Q-Sepharose column.

Initial attempts to determine the molecular weight of the native enzyme was via gel filtration chromatography on a matrix of Sephacryl S-400. Surprisingly, chromatography of MCCase in this column led to the loss of activity. In only two attempts less than 5 % of the activity were recovered by which we were able to determine a molecular weight of 1,400,000 (Fig. 3). Analysis of the fractions eluted from these

columns by SDS-PAGE and Western blotting with ^{125}I -streptavidin revealed that the biotin subunit of MCCase was eluting in fractions corresponding to molecular weights ranging from 1,400,000 to 80,000 (Fig. 4). We suspected that the enzyme was dissociating during chromatography. Indeed, we have observed similar behavior by the purified maize MCCase when it was chromatographed over the same matrix.

To obtain a more reliable determination of the molecular weight of the native MCCase we utilized two electrophoretic procedures. In one procedure the MCCase preparation was subjected to electrophoresis in an polyacrylamide gel composed of a linear gradient of acrylamide (2-16 %). Electrophoresis was carried out until MCCase and the standard proteins of known molecular weight caused migrating due to their reaching the gel pore size corresponding to each proteins stokes radius. The standard proteins were detected by staining of the gel with Coomassie Brilliant Blue (Fig. 5A), and MCCase was detected with ^{125}I -streptavidin (Fig. 5B) or anti-MCCase serum (Fig. 5C) after Western transfer. Comparison of the migration distance of MCCase with the migration distances of the standard proteins is illustrated as a standard curve in Fig. 5D. This procedure yielded a molecular weight of 1,120,000 for the native MCCase.

The second electrophoretic procedure which was used measured the rate of migration of MCCase during electrophoresis in a polyacrylamide gel composed of a linear gradient of 2-16 % acrylamide. Comparison of the rate of migration of MCCase with the rate of migration of standard

proteins enabled us to determine the molecular weight of MCCase. The MCCase preparation and the mixture of standard proteins of known molecular weight were loaded into separate wells of a slab gel at intervals of about 2¹/₂ hours. After electrophoresis for 12 h, the portion of the gel containing the standard proteins was stained with Coomassie Brilliant Blue (Fig. 6B), and the portion of the gel containing MCCase was transferred electrophoretically to nitrocellulose and MCCase was detected with ¹²⁵I-streptavidin (Fig. 6A). The plot of the square root of time of electrophoresis against distance migrated by MCCase and by each of the molecular weight standards is shown in Fig. 6C. This plot generates a series of linear lines whose slopes are proportional to the molecular weight of the proteins. A logarithmic plot of the molecular weight of each protein versus the slope of the line generated in Fig. 6C is shown in Fig. 6D, which illustrates the relationship between molecular weight and rate of migration. The molecular weight of tomato MCCase was estimated from this curve as 1,200,000.

Immunochemical Studies of MCCase — We have isolated cDNAs coding for the biotin-containing subunit of tomato MCCase (Wang et al. submitted). The amino acid sequence of this subunit deduced from the nucleotide sequences of the cDNA clones was compared with the primary sequences of other biotin-containing enzymes. This comparison identified two domains within the MCCase sequence that are homologous to other biotin enzymes. These structurally homologous domains represent the biotin carboxylase and biotin

carboxyl carrier functional domains that are required for catalysis of the first-half reaction catalyzed by biotin-dependent carboxylases. The biotin carboxylase and biotin carboxyl carrier domains are separated by a stretch of 170 amino acid residues which shows low homology to other biotin enzymes. Indeed, comparison of the corresponding regions of the propionyl-CoA carboxylase with acetyl-CoA carboxylase sequences indicates that the low homologies between these two additional enzymes are maintained in this region. The lack of conservation in the amino acid sequence between the biotin carboxylase and biotin carboxyl carrier domain may indicate that this region of the enzyme is not structurally important for catalysis.

To test this hypothesis we expressed three regions of the MCCase sequence as chimeric proteins in *E. coli*. These three regions are names BX1, BX3 and BTN (240-322, 368-436, and 217-436 respectively shown in Fig. 7). BX1 is located in between of biotin carboxylase domain and biotin carboxyl carrier domain, where is the region of lack of amino acid sequence homology in biotin enzymes. BX3 is the putative biotin carboxyl carrier domain whose sequence is relatively conserved in biotin enzymes. BTN is the region contains both BX1 and BX3. Each chimeric protein was purified from *E. coli* extracts and utilized as antigen for the immunization of rabbits. The resulting antisera were individually utilized to dissect the functional significance in catalysis of each region of the MCCase sequence.

All three antisera reacted equally with the biotin subunit of MCCase as judged by utilizing them to detect the MCCase subunit. Each of these

antisera were then tested for their ability to inhibit MCCase activity. Anti-BTN and anti BX3 sera were capable of inhibiting MCCase activity. Both these antisera were generated to epitopes that include the biotin carboxyl carrier domain. In contrast, anti-BX1 serum could not inhibit MCCase activity. This later antiserum was generated to epitopes containing the nonconserved region between the biotin carboxylase and biotin carboxyl carrier domain. These immunological studies are consistent with the hypothesis that the region of the biotin subunit of MCCase, between the biotin carboxylase and biotin carboxyl carrier domains (residue 240-322 of T-mcc in Fig. 7), that is not highly conserved among biotin enzymes, does significantly or directly contribute to the structure and function of the active site(s) of MCCase.

Immunological Comparison of MCCases— The antisera generated to the biotin subunit of the tomato MCCase was utilized to investigate the similarity in the epitopes of MCCase in different species. Cell-free extracts were prepared from maize seeds and leaves, soybean seeds and leaves, tobacco leaves, tomato leaves and bovine heart. In addition, proteins were extracted from acetone powders prepared from bovine kidneys and livers. Aliquots of these extracts were fractionated by SDS-PAGE and proteins were electrophoretically transferred to nitrocellulose filters. These filters were incubated with ^{125}I -streptavidin to detect biotin-containing polypeptides (Fig. 8A) or they were incubated with antisera to the biotin subunit of MCCase (Fig. 8B). The results indicated that the antiserum of the biotin-containing subunit of tomato MCCase reacted with a 83 kDa biotin-containing

polypeptide, the biotin-containing subunit of MCCase, from maize seed. The antiserum also reacted with a 83 kDa biotin-containing polypeptide of soybean leaves. This 83 kDa biotin-containing polypeptide from soybean has been shown to be the large subunit of MCCase from soybean (Song et al., submitted). The antiserum of tomato MCCase reacted with two biotin-containing polypeptides of 78 kDa and 83 kDa from tobacco leaves. The identities of these two biotin-containing polypeptides were still not known. The immunological cross-reaction suggested they had similar structural features. Comparison of their sizes with tomato MCCase and soybean MCCase suggested both of them might be MCCase isozymes. The antiserum of tomato MCCase did not cross-react with bovine MCCase even after the autoradiograph was exposed for a longer time.

DISCUSSION

The subunit composition of the tomato MCCase was investigated by affinity purifying the enzyme from a preparation in which MCCase was the sole detectable biotin-containing protein. This purification resulted in the isolation of two polypeptides, one of which contained biotin (78 kDa), and the second did not contain biotin (60 kDa). Since isolation of the 60 kDa, biotin-free polypeptide depended on the biotin prosthetic group of the enzyme, we concluded that these two proteins are in a nondissociable complex, the MCCase holoenzyme. In addition, these two polypeptides occurred in equal molar stoichiometry. Thus the tomato MCCase appears to be heteromeric, being composed of equal number of two different types of subunits.

This molecular arrangement of the tomato MCCase is similar to MCCases from wide variety of species. MCCase from bovine kidney (Lau et al., 1980), *Achromobacter* (Apitz-Castro et al., 1970), *Pseudomonas citronellas* (Fall and Hector, 1977), carrots (Yang et al., 1993), pea (Alban et al., 1993), potato (Alban et al., 1993), maize (Diez et al., submitted) and soybean (Song et al., submitted) all are composed of two types of subunits; the larger, of between 73 kDa and 96 kDa contains biotin, and a smaller subunit of between 53 kDa and 78 kDa, is biotin-free.

These MCCases appear to present two types of quaternary structure. The enzyme from *Achromobacter*, *P. citronellas*, Pea and potato have an $\alpha_4\beta_4$ structure, whereas the enzyme from bovine kidney, carrot, maize

and soybean appear to have an $\alpha_6\beta_6$ structure. The determination of these quaternary structures are very dependent upon the determination of the molecular weight of the native MCCase. All these enzymes are very large complexes, ranging in size from 500 kDa to nearly 1,000 kDa, and these molecular weight determinations were because of a lack of an appropriately sized standard protein, the result of extrapolation of a standard curve. It is thus unclear whether the purported difference between $\alpha_4\beta_4$ and $\alpha_6\beta_6$ structures is a real difference in these enzymes or a result of errors in determination of the molecular weights of the enzymes.

In the case of the tomato MCCase, the native enzyme is obviously a large complex; determinations of the molecular weight of the native enzyme using three different techniques gave values ranging between 1,120 kDa and 1,400 kDa. For each determination estimation of the molecular weight of the enzyme was via extrapolation of a standard curve. However, it is clear from our data that the tomato MCCase is large than apoferritin dimer ($M_r = 886,000$), which indicates that tomato MCCase has an $\alpha_n\beta_n$ quaternary structure where $n \geq 6$. But to draw a definite conclusion of the quaternary structure of the tomato MCCase requires additional experiments.

The reactions catalyzed by biotin enzymes can be divided into two half-reactions and each half-reaction is catalyzed at structurally distinct subsites. The structural organization of these subsites appear to have evolved via a gene-fusion mechanism. Thus, whereas for the *E. coli* acetyl-CoA carboxylase the biotin carboxylase and

carboxyltransferase subsites and the biotin carboxyl carrier domain are coded by separate genes and reside on separate proteins, in the eukaryotic acetyl-CoA carboxylase (rat, chicken, yeast) these two subsites and the biotin carboxyl carrier domain all occur on a single gene product. Propionyl-CoA carboxylase and MCCase appear to be intermediates in this evolutionary scheme, since the biotin carboxylase subsite and the biotin carboxyl carrier domain occur on one subunit of these enzymes and the carboxyltransferase subsite is on the second subunit. With the recent cloning of genes and cDNAs coding for some of these proteins, these conserved domains can be recognized by sequence homologies.

By using antisera which were generated to portions of the biotin subunit of MCCase we were able to distinguish a region of this subunit which appears to be nonessential to catalytic function. This region, between residue 240 to 322, divides the biotin carboxylase subsites from the biotin carboxyl carrier domain, and shows low sequence homology to other biotin enzymes. The low sequence identity between this domain and other biotin enzymes could be interpreted to mean that this region does not contribute to structures essential for catalytic function. This sequence may provide a structural frame to orient function domains to be in proper conformation as proposed by Al-Feel (Al-Feel et al., 1992), or was flexible in biotin enzymes. The later possibility is more consistent with the gene-fusion hypothesis (Lynen, 1979) since when gene fuses together during evolution the sequence between function domains might come randomly. It is obvious that

random cassette mutagenesis experiments in this sequence segment may clarify our understanding.

Finally, the antiserum directed against the biotin subunit of the tomato MCCase was utilized to probe the immunological similarity of MCCase in different plant species and in bovine tissues. These analyses indicate that the plant MCCases share common epitopes that are recognized by the antibodies in the serum used. However, these antibodies were unable to detect the bovine MCCase, at least by the Western procedure which was utilized. Thus during evolution the animal and plant MCCase appear to have acquired sufficient structural differences, so as to lose common epitopes. We had previously obtained evidence for functional differences between animal and plant MCCases, by the discovery that acetoacetyl-CoA is a potent competitive inhibitor of the plant MCCase, whereas it is an alternative substrate of the animal MCCase (Diez et al., submitted)

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FIG. 1. Partial Purification of MCCase . *Panel A*, MCCase was extracted from leaves of tomato and precipitated with PEG as described in the Materials and Methods. The PEG precipitated fraction was subjected to anion exchange chromatography on a Q-Sepharose column. MCCase was eluted from the Q-Sepharose column with a linear gradient of increasing KCl concentration (—), and MCCase activity was determined in the eluate (—◇—). *Panel B*, MCCase containing fractions obtained from the Q-Sepharose column were pooled. An aliquot of the leaf extract (Lane 1), and an aliquot of the partially purified MCCase preparation (Lane 2) were analyzed by SDS-PAGE and Western blot analysis. The blot was incubated with ¹²⁵I-streptavidin to detect biotin-containing polypeptides.

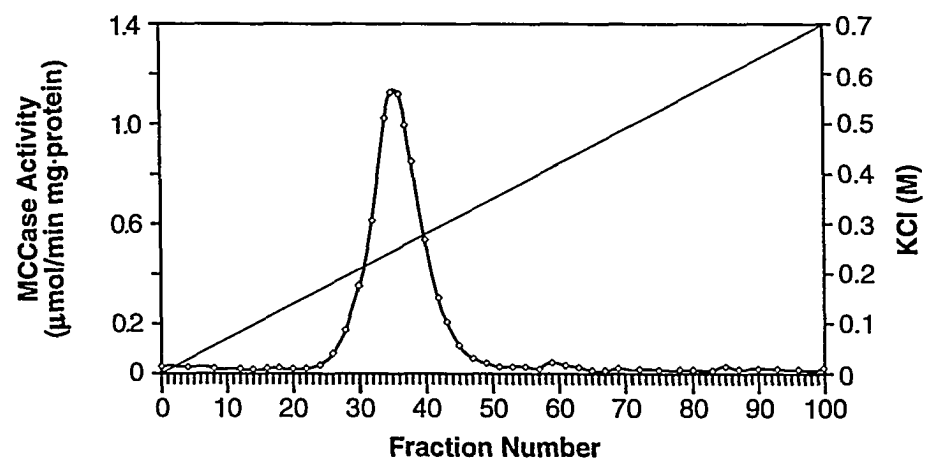
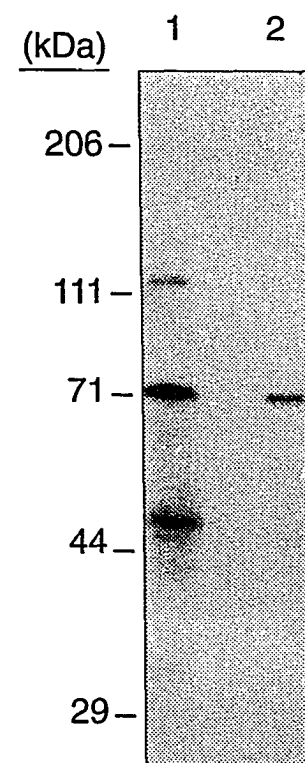
A**B**

FIG. 2. MCCase Subunit Composition. The partially purified MCCase preparation, obtained by anion-exchange chromatography, was subjected affinity chromatography with avidin-agarose (A-agarose) (Lane 1 to 3), or with avidin-agarose pretreated with 2 mM biotin (A-agarose + biotin) (Lane 4 to 6), as described in the Materials and Methods. Aliquots of the partially purified MCCase preparation (Lane 1, 4), the avidin-nonbinding fraction (Lane 2, 5), and avidin binding fraction (Lane 3, 6) were subjected to SDS-PAGE. The resulting gel was silver stained (Panel A), or subjected to Western blot analysis. The blot was incubated with ^{125}I -streptavidin to detect biotin-containing polypeptides.(Panel B).

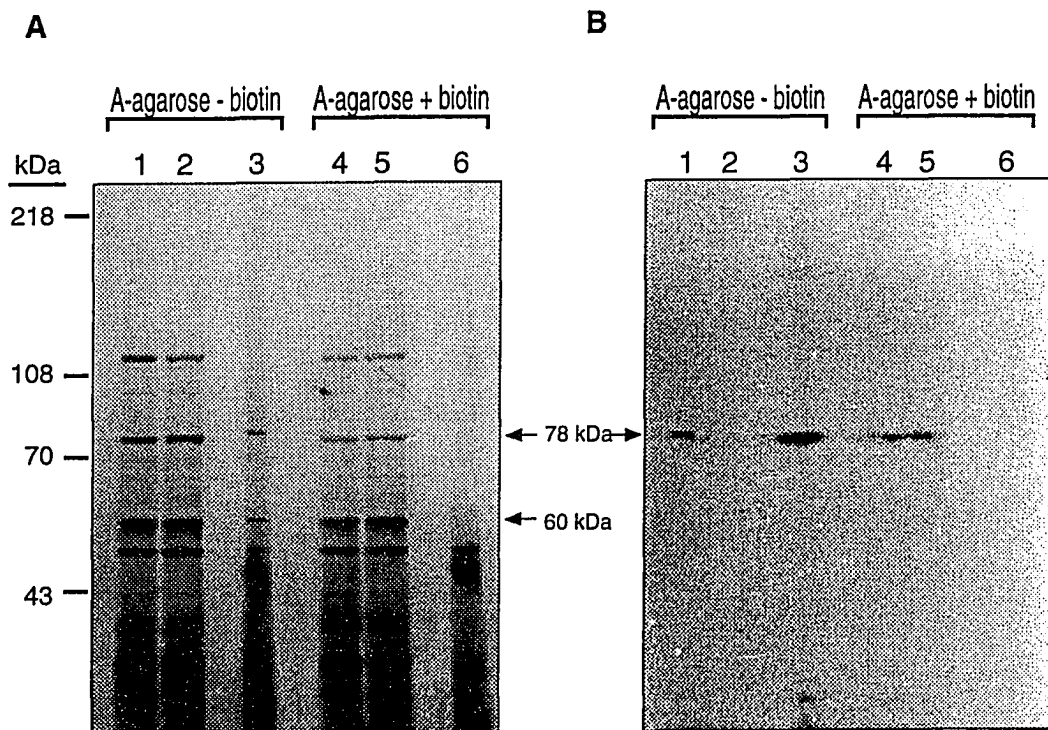


FIG. 3. Determination of the Molecular Weight of the Native MCCase by Gel Filtration Chromatography. An aliquot of the partially purified MCCase preparation subjected to gel filtration chromatography on a column of Sephacryl S-400 (1.5 cm x 100 cm). The eluate from the column was monitored for MCCase activity. Elution from the column was calibrated by chromatography of the molecular weight standard proteins: apoferritin (886 kDa dimer, 443 kDa monomer), bovine thyroglobulin (669 kDa), and yeast alcohol dehydrogenase (150 kDa).

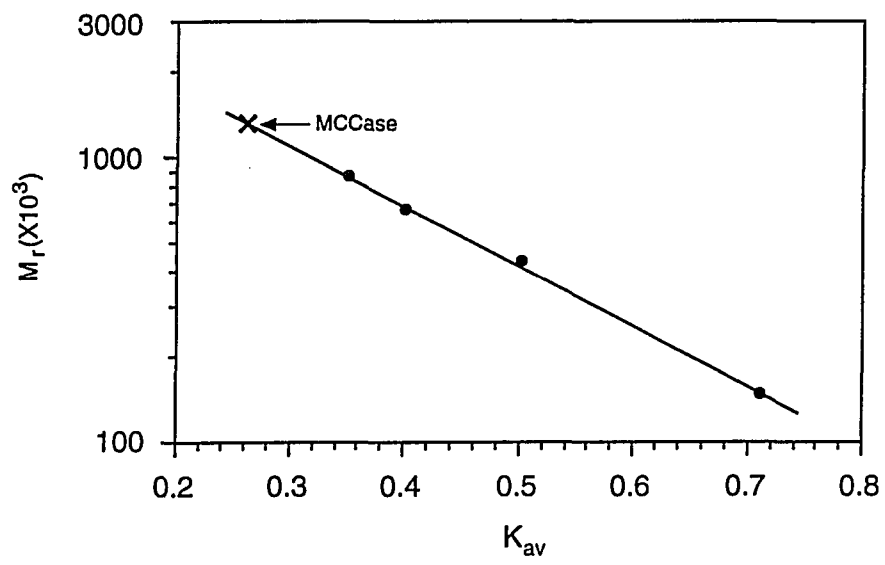


FIG. 4. Elution of the MCCase Biotin Subunit from a Sephacryl S-400 Column. Aliquots of every fifth fraction of the elution from the Sephacryl S-400 column shown in Fig. 3, were subjected to SDS-PAGE and subjected to Western blot analysis. The blot was incubated with ^{125}I -streptavidin to detect the biotin subunit of MCCase. The fractions corresponding to the elution volume of the molecular weight standard proteins are labeled.

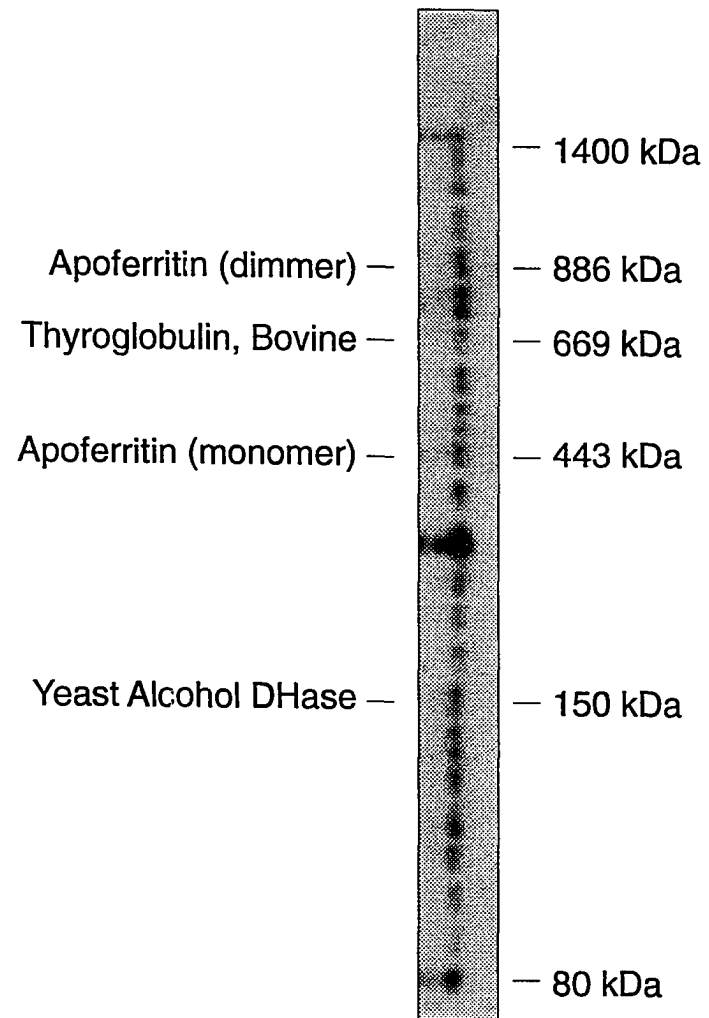


FIG. 5. Determination of the Molecular Weight of the Native MCCase by Nondenaturing Polyacrylamide Gel Electrophoresis.

An aliquot of the partially purified MCCase preparation and molecular weight standard proteins were subjected to electrophoresis in a gel composed of a linear gradient of 2-16% acrylamide. After electrophoresis at 200 volts for 45 h (Lane 1), or 48 h (Lane 2), the molecular weight standard proteins were visualized by staining with Coomassie Brilliant Blue (Panel A), and MCCase was detected by Western blot analyses of the resulting gels. The blots were sequentially incubated with the antiserum to the biotin subunit of MCCase and ^{125}I -Protein A (Panel B), or with ^{125}I -streptavidin (Panel C). The molecular weight of MCCase was determined by comparing the migration of the enzyme with the migration of the molecular weight standard proteins (Panel D). Molecular weight standard proteins were: apoferritin (886 kDa dimer, 443 kDa monomer), urease (545 kDa dimer, 272 kDa monomer), and bovine albumin (132 kDa dimer, 66 kDa monomer).

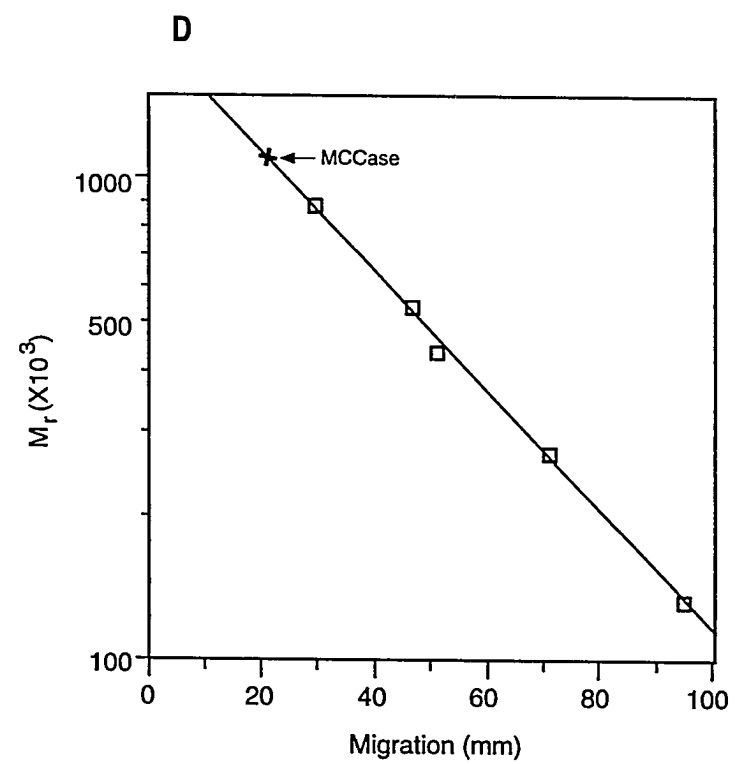
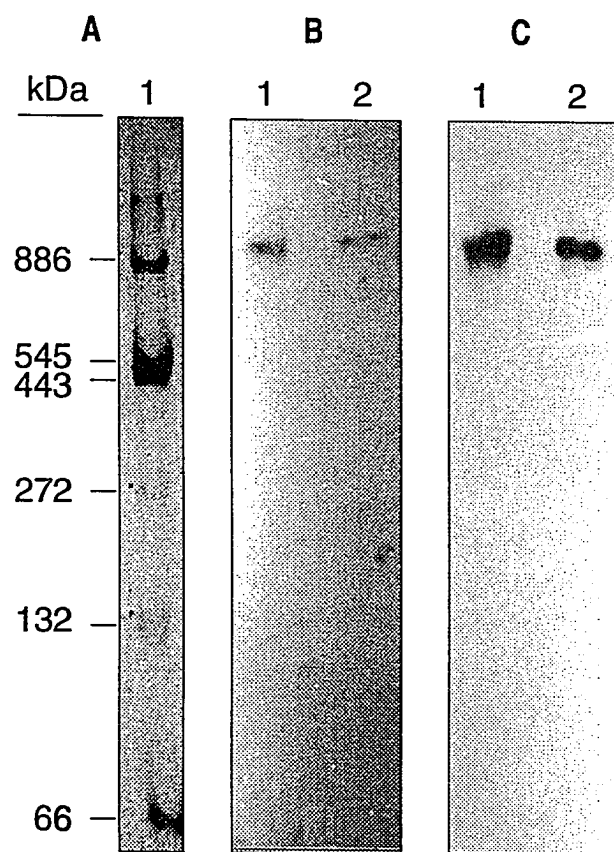


FIG. 6. Determination of the Molecular Weight of the Native MCCase by the Rate of Migration in Polyacrylamide Gel Electrophoresis.

An aliquot of the partially purified MCCase preparation and molecular weight standard proteins were subjected to electrophoresis in a gel composed of a linear gradient of 2-16% acrylamide for a period of 2.1 h (Lane 1); 4.8 h (Lane 2); 7.4 h (Lane 3); 9.5 h (Lane 4); 11.7 h (Lane 5). MCCase was detected by Western blot analysis of the resulting gel, and incubation of the blot with ^{125}I -streptavidin (Panel A). The molecular weight standard proteins were visualized by staining with Coomassie Brilliant Blue (Panel B). The migration distance of the standard proteins and MCCase were plotted against the square root of the time of electrophoresis (Panel C). The logarithm of the slopes of the lines obtained in Panel C was plotted against the logarithm of the molecular weight of the standard proteins (Panel D). The molecular weight of MCCase was extrapolated from this plot. Molecular weight standard proteins were apoferritin (886 kDa dimer, 443 kDa monomer), bovine albumin (132 kDa dimer, 66 kDa monomer), and chicken albumin (45 kDa).

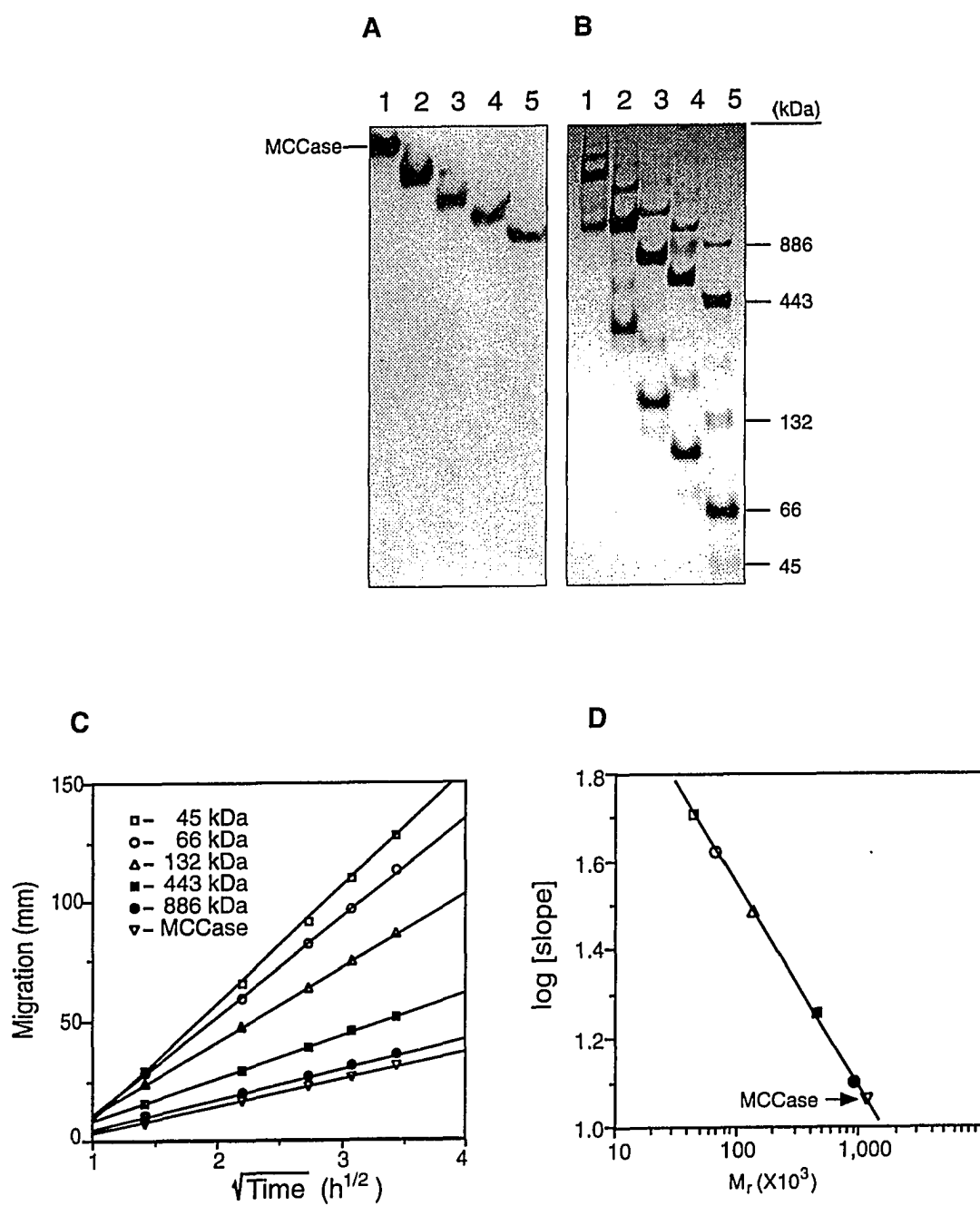


FIG. 7. Preparation of Domain-Specific Antisera. The amino acid sequence of the biotin subunit of tomato MCCase (MCC-1) (Wang et al., submitted), can be partitioned by sequence homology into two potential functional domains, the biotin carboxylase domain (residues 1-191) and the biotin carboxyl carrier domain (residues 353-436). These two domains are separated by a region that shows low homology among all biotin enzymes. Three regions of the MCC-1 sequence were expressed in *E. coli*, as fusion proteins. The BTN region (residues 217-436) was expressed as a glutathione S-transferase fusion; the BX1 (residues 240-322) and BX3 (residues 368-436) regions were expressed as β -galactosidase fusion proteins. Antisera were generated to each of these expressed fusion proteins.

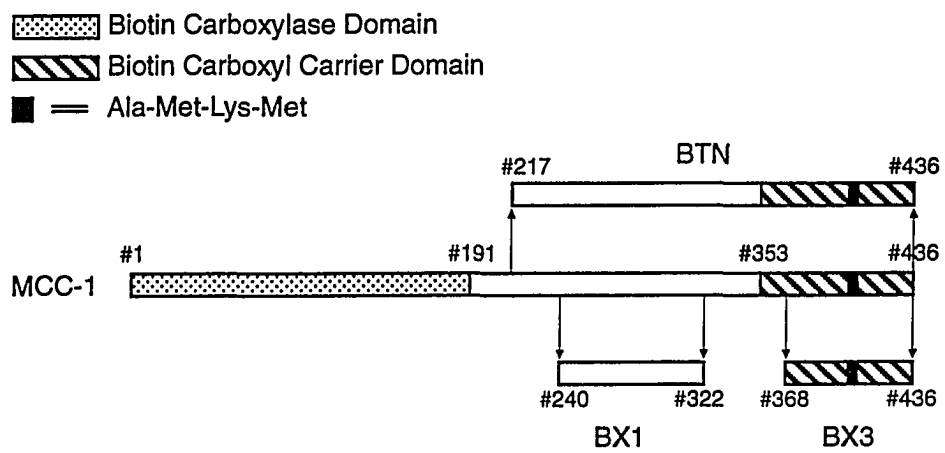
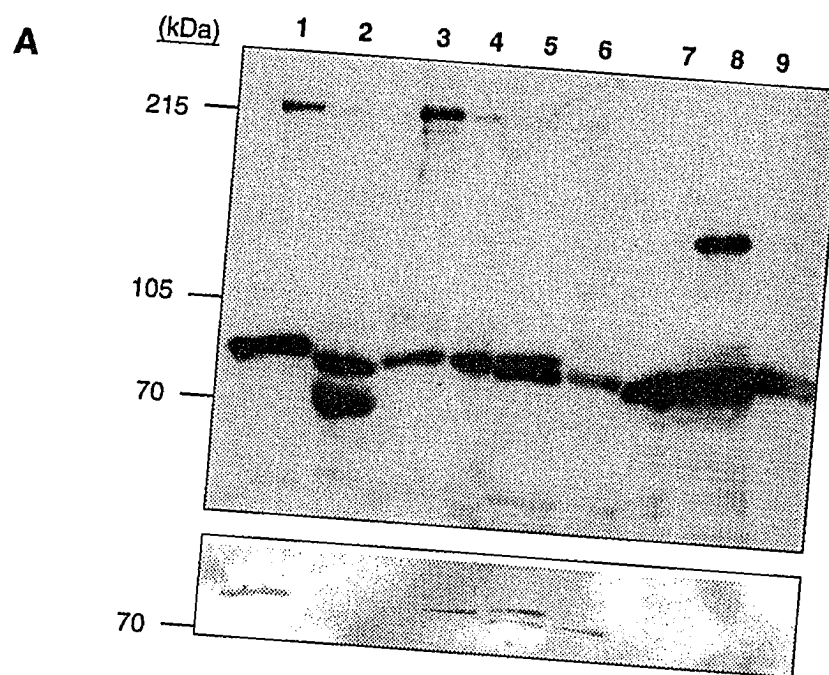


FIG. 8. Immunological Cross-Reactivity of the Biotin Subunit of MCCase. Aliquots containing equal amount of protein extracted from seeds of maize (Lane 1) and soybean (Lane 2), leaves of maize (Lane 3), soybean (Lane 4), tobacco (Lane 5), and tomato (Lane 6), bovine heart (Lane 7), kidney (Lane 8) and liver (Lane 9) were subjected to SDS-PAGE and Western blot analyses. The resulting blot was incubated with ^{125}I -streptavidin (Panel A), or sequentially incubated with the antiserum to the biotin subunit of tomato MCCase and ^{125}I -Protein A (Panel B).



**PAPER III. REGULATION OF β -METHYLCROTONYL-CoA
CARBOXYLASE ACTIVITY BY BIOTINYLATION
OF THE APOENZYME**

**REGULATION OF β -METHYLCROTONYL-CoA CARBOXYLASE
ACTIVITY BY BIOTINYLATION OF THE APOENZYME**

Xun Wang¹, Eve Syrkin Wurtele² and Basil J. Nikolau^{1†}

¹Department of Biochemistry and Biophysics, and ²Department of Botany, Iowa State University, Ames, Iowa 50011

†Corresponding Author: Dr. Basil J. Nikolau
Department of Biochemistry & Biophysics,
Iowa State University,
Ames, IA 50011
Phone: (515)294-9423
FAX: (515)294-0453

Running Title: Regulation of β -methylcrotonyl-CoA carboxylase

¹Abbreviations:

BSA, bovine serum albumin; E-64, L-*trans*-epoxysuccinyl leucylamido(4-guanidino)-butane; EtBr, ethidium bromide; HEPES, N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; MCCase, β -methylcrotonyl-CoA carboxylase; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris-HCl, tris[hydroxymethyl]aminomethane hydrochloride

ABSTRACT

The distribution of β -Methylcrotonyl-CoA carboxylase among organs of tomato was studied. The enzyme is found in all organs examined, but its specific activity in each organ appears to be inversely proportional to the photosynthetic competence of that organ. This finding is consistent with the hypothesis that the metabolic function of MCCase is for the catabolism of leucine, from which carbon can be respired to generate ATP and reducing equivalents, when these cannot be generated directly from photosynthesis. The 10-fold difference in specific activity of this enzyme in leaves and roots could not be attributed to differential accumulation of the biotin subunit of MCCase or the mRNA coding for that subunit. Instead, this difference in MCCase activity between roots and leaves appears to be due to differential biotinylation of the enzyme. Thus the lower activity of MCCase in leaves is due to the lower degree of biotinylation of the biotin subunit of the enzyme. This discovery was further substantiated by the demonstration that a pool of apoenzyme exists in tomato leaves. The under-biotinylation of this enzyme in leaves is probably not due to the lack of biotin in that organ, but probably reflects the differential regulation of the biotinylation reaction by a mechanism that is yet to be investigated.

INTRODUCTION

β -Methylcrotonyl-CoA carboxylase (¹MCCase; EC 6.4.1.4) is a biotin-containing enzyme that catalyzes the ATP-dependent carboxylation of β -methylcrotonyl-CoA (MC-CoA) to form β -methylglutaconyl-CoA (MG-CoA). MCCases from bacteria and animals have been extensively investigated (Schiele and Lynen, 1981; Fall, 1981; Lau and Fall, 1981), and its role in the catabolism of leucine has been well established in these systems (Moss and Lane, 1971). In addition, MCCase may have a metabolic function in the "mevalonate shunt" by which mevalonate can be metabolized to non-isoprenoid compound (Edmond and Popjak, 1974; Edmond et al., 1976).

By comparison, knowledge of this enzyme from plants is limited. Indeed, MCCase activity was first reported in plant cell-free extract in 1990 (Wurtele and Nikolau, 1990). More recently, the presence of MCCase in the plant kingdom was confirmed by purification of the enzyme (Yang et al., 1993; Alban et al., 1993; Claude et al., 1993; Diez et al., submitted) and isolation of the genes and cDNAs coding for its biotin subunit (Wang et al., submitted; Song et al., submitted).

Elucidation of the metabolic function of MCCase in plants is still to be established. It undoubtedly plays a role in leucine catabolism, however, the potential functioning of an alternate pathway for leucine catabolism in peroxisomes, not requiring MCCase (Gerbling and Gerhardt, 1989), needs to be explored. In addition, the significance of leucine catabolism to the plant's physiological status is unknown.

As an initial investigation to address some of these questions we undertook an investigation of the regulation of the organ-specific expression of MCCase in tomato. These studies led to the discovery of a novel, post-translational mechanism by which the organ-specific difference in MCCase activity is established, namely differential biotinylation of the apoenzyme.

MATERIALS AND METHODS

Materials — β -Methylcrotonyl-CoA, d-biotin, E-64, PMSF, HEPES, biotinyl BSA, NP-40, streptavidin, biotinyl alkaline phosphatase and *p*-nitrophenyl phosphate were purchased from Sigma Chemical Co. Other chemicals were purchased from Fisher Scientific Inc. Radioisotopes were purchased from Amersham. Avidin-agarose was purchased from Pierce. Bio-Gel P-6DG was purchased from Bio-Rad Laboratories. Nitrocellulose was purchased from MSI.

Tomato (*Lycopersicum esculantus* var. *Rutgers*) were grown in a greenhouse at 18-22 °C under supplemented illumination with a 15 hr day period. Leaves and roots were harvested 60-80 days after planting.

Enzyme Extraction — Two types of extracts were prepared utilizing non-denaturing or denaturing conditions. In both instances plant materials were frozen in liquid nitrogen and ground in a mortar with a pestle while the tissue was still frozen. For a non-denatured extract, the frozen powder was homogenized after addition of two volumes of pre-chilled (0 °C) 100 mM HEPES-KOH (pH 7.0), 1 mM EDTA, 20 mM 2-mercaptoethanol, 100 μ g/ml PMSF, 1 mM E-64, 20% (v/v) glycerol, 0.1% (v/v) Triton X-100. After the extract thawed (at 4 °C), it was filtered through four layers of cheesecloth and then the filtrate was centrifuged at 12,000 *g* for 10 min. Low molecular weight molecules were removed from the supernatant by passing 1 ml of such extract through a 5 ml Bio-Gel P-6DG gel filtration column.

For the preparation of a denatured extract two volumes of an SDS-containing extraction buffer, which contained 100 mM Tris-HCl (pH 7.0), 10 mM DTT, 5 mM EDTA and 2% (w/v) SDS were added to the powder. The mixture was homogenized further at 4 °C until it was thawed. The extract was transferred to a glass tube and heated in a bath of boiling water for 15 min. The mixture was then clarified by centrifugation at 10,000 *g* for 10 min.

Purification of biotin-containing polypeptides — An SDS-denatured protein extract was diluted with 9 volumes of 50 mM Tris-HCl (pH 7.0), 1 mM EDTA to reduce the SDS concentration to 0.2% (w/v). Biotin-containing polypeptides were purified by avidin-agarose affinity batch chromatography. For every 5 grams of plant material from which the extract was prepared, 2.5 ml avidin-agarose was used. The mixture was incubated on a shaker at 4 °C for 40 min. Nonbinding proteins were removed by washing the matrix with 250 ml of wash solution [50 mM Tris-HCl (pH 7.0), 1 mM EDTA, 0.2% (w/v) SDS]. Finally biotin-containing polypeptides were eluted from the avidin-agarose by suspending the matrix in 100 mM Tris-HCl (pH 7.0), 1 mM EDTA, 2% (w/v) SDS and incubating the mixture in a bath of boiling water for 15 min. The matrix was removed by centrifugation at 4,000*g* for 10 min and the supernatant retained.

Protein Analysis — SDS-PAGE was carried out as described (Laemmli et al., 1970). Proteins were visualized either by staining with Coomassie Brilliant Blue or by silver staining.

For Western analysis, proteins were transferred from the gel to nitrocellulose filters with a semi-dry trans blot apparatus according to the manufacturer's instructions (PolyBlot, American Bionetics, Inc.). Immunological detection of proteins was conducted with antiserum diluted between 1:200 to 1:1000 with a 3% (w/v) BSA solution, following which, antigen-antibody complexes were detected with ^{125}I -protein A. Biotin-containing polypeptides were detected with ^{125}I -streptavidin (Nikolau et al., 1985).

Protein concentration was determined by the Bradford method (Bradford, 1976) using the Bio-Rad protein assay reagents. BSA was used as a standard.

MCCase Assay — MCCase activity was measured as the rate of incorporation of radioactivity from the substrate $\text{NaH}^{14}\text{CO}_3$ into the acid-stable product (Wurtele and Nikolau, 1990).

Antiserum — Antiserum against the biotin-containing subunit of MCCase of tomato was prepared as described before (Wang et al., submitted). In brief, the cDNA coding for the biotin-containing subunit of MCCase of tomato was expressed in *E. coli*. The affinity purified expressed protein was used as an antigen for the generation of antiserum in New Zealand White female rabbits.

Isolation and Analysis of RNA — Total cellular RNA was isolated from tomato leaves and roots, as previously described by Logemann et al. (1987). RNA was fractionated by electrophoresis in a 1.2% (w/v) agarose gel containing formaldehyde as described (Lehrach et al.,

1977). The fractionated RNA was transferred to a nylon membrane (MSI) with 10 X SSC and hybridized to a ^{32}P -labeled cDNA fragment. Hybridization was carried out as recommended by manufacture (Micron Separations Inc. MA). The blots were washed with three 20 min washes in 0.1 X SSC, 0.5% SDS at 65 °C.

Extraction of Biotin — Tomato leaves or roots were frozen in liquid nitrogen and powdered with a mortar and pestle. The powder was then further homogenized with two volumes of 10% (w/v) trichloroacetic acid (TCA). The mixture was kept on ice for 30 min and then clarified by centrifugation at 12,000 *g* for 25 min. Free biotin was recovered in the supernatant. Protein-bound biotin was recovered in the pellets and was released by resuspending the pellet in 2 M H_2SO_4 and was incubating the mixture in an oven at 120 °C for 2 hr. Both free-biotin and protein bound-biotin solutions were neutralized with 5 N NaOH and clarified by centrifugation for 10 min at 12,000 *g*. The supernatants were subsequently used for determination of the biotin content.

To determine the concentration of total biotin in tissue, plant materials were frozen in liquid nitrogen and after homogenization further homogenized in two volumes of 2 M H_2SO_4 . The resulting mixture was heated at 120 °C for 2 hr and then neutralized with 5 N NaOH. Biotin content was determined after the insoluble materials were removed by centrifugation.

In the parallel control experiments, 40 ng of d-biotin was added as an internal standard to each 0.5 grams sample of plant material that was extracted prior to the H_2SO_4 . treatment.

Biotin Assay — Biotin concentration was determined by a procedure based on competition for binding to streptavidin (Bayer et al., 1990). In brief, the wells of microtiter plates were coated with biotinylated BSA and subsequently quenched with BSA. Streptavidin was then added to each well at a concentration of 5 $\mu\text{g/ml}$ and the plates were incubated at room temperature for 30 min. The excess streptavidin was removed by washing the wells with PBS (7.4). The bound streptavidin in the microtitre wells was incubated with either different standard concentrations of biotin or serial dilution of the unknown samples. Following a 30 min incubation at room temperature the wells were washed and 1 unit/ml biotinylated alkaline phosphatase was added to each well. After a 30 min incubation at room temperature, excess biotinylated alkaline phosphatase was removed by washing the wells. Bound alkaline phosphatase was incubated with 1 mg/ml *p*-nitrophenyl phosphate at room temperature for one to three hours. A plot was obtained by plotting the absorbance (405 nm) of the mixture from each well versus biotin concentration applied to the well.

RESULTS AND DISCUSSION

Distribution of MCCase Among Different Organs of Tomato — To begin to ascertain the metabolic significance of MCCase in plants, and to determine how MCCase maybe regulated, we determined the distribution of MCCase among different organs of tomato. Crude extracts were prepared from leaves, stems, roots, ripening fruits and ripe fruits of tomato, and MCCase activity was measured. MCCase activity was found in all the organs examined (Table 1). Its specific activity is highest in roots, followed in descending order by ripe fruits, ripening fruits, stems, and leaves. The specific activity of MCCase is about ten times higher in roots than in leaves.

Comparison of MCCase Gene Expression Between Leaves and Roots — Because of the extreme difference in MCCase activity between leaves and roots, we undertook a comparative study of these organs to ascertain the molecular mechanism by which this difference is established. In doing so we expected to unveil a potential mechanism by which MCCase activity is regulated.

Initial experiments were aimed at eliminating a trivial explanation for the difference in MCCase activity between leaves and roots, namely the presence of an inhibitor of MCCase in leaves, which upon extraction of the tissue, bound to the enzyme and caused an underestimation of MCCase activity in the extracts. To eliminate this potential explanation, leaf and root extracts were mixed and subsequently

assayed for MCCase activity. MCCase activity from each tissue was additive and thus no evidence for the presence of an inhibitor was found (data not shown).

To ascertain whether the differences in the specific activity of MCCase between leaves and roots was due to different levels of accumulation of the biotin subunit of MCCase, we performed Western blot analyses using the antibody specific to this subunit of the enzyme. Aliquots of crude extracts from leaves and roots containing equal amounts of protein were fractionated by SDS-PAGE. The resulting gel was subsequently analyzed by Western transfer and the biotin subunit of MCCase was immunologically detected and quantitated. Surprisingly, densitometric scans indicated the intensity of the protein band was about equal in the extracts from roots and leaves (Fig. 1C). Consistent with the nearly equal accumulation of the biotin subunit of MCCase in leaves and roots, we found that the accumulation of the mRNA coding for this subunit to be at equal levels in both organs (Fig. 1B). These findings lead us to conclude that the ten-fold difference in MCCase activity between leaves and roots is not due to differences in gene expression. Both the steady state mRNA levels and the steady state levels of the biotin subunit of MCCase are equal in both organs. Therefore, the enzyme that accumulated in roots appears to be catalytically more efficient than the enzyme which accumulated in leaves.

Differential Biotinylation of MCCase in Roots and Leaves — The catalytic efficiency of an enzyme can be regulated by a number of

mechanisms, one of which is post-translational modification. In the case of MCCase, one post-translational modification reaction that is essential for enzyme activity is biotinylation. To test whether differential biotinylation of MCCase in roots and leaves maybe the cause of the difference in the activity of MCCase in these organs, extracts from each organ were subjected to Western blot analysis probed with ^{125}I -streptavidin. This analysis demonstrates that the biotin subunit of MCCase is biotinylated to a higher degree in roots than in leaves. Indeed, the intensity of the protein band revealed by ^{125}I -streptavidin was 10- to 12-fold higher in roots than in leaves (Fig. 1D), consistent with the difference in MCCase activity. These results were confirmed by Western blots of root and leaf extracts in which aliquots containing equal amounts of MCCase activity were subjected to analysis. In these analyses, The biotin prosthetic group on the MCCase biotin subunit was detected by ^{125}I -streptavidin. Both leaf and root MCCases were found to contain equal amount of the biotin prosthetic group (Fig. 1E), in direct correlation with the amount of MCCase activity loaded on the blot.

The finding that the different levels of MCCase activity in roots and leaves is caused, in part by differential biotinylation of the enzyme leads us to predict that a pool of apo-MCCase is present in tomato leaves. This pool of apo-MCCase was identified by the experiment illustrated in Fig. 2. An SDS-denatured protein extract prepared from tomato leaves was chromatographed over an avidin-agarose affinity matrix to remove all the biotin-containing polypeptides from the

extract. The biotin-containing polypeptides were subsequently eluted from the matrix with boiling 2% SDS. An aliquot of the original extract, and the fraction containing biotin-containing polypeptides, free of biotin polypeptides, were fractionated by SDS-PAGE and the resulting gel was subjected Western analyses. There were no detectable biotin-containing polypeptides in the avidin nonbinding fraction as revealed by ^{125}I -streptavidin. However, the antisera to the biotin subunit of MCCase detects the presence of this subunit in the avidin nonbinding fraction (Fig. 2A, Lane 2). This immunologically detectable subunit of MCCase does not contain biotin and represents the pool of apo-MCCases in tomato leaves.

Biotin Content in Leaves and Roots — The different biotinylation status of MCCase in leaves and roots of tomato may be a consequence of the availability of biotin in each of these organs, in which case this potential regulatory mechanism would not be specific to MCCase but would be common to all biotin enzymes. This phenomenon has been previously observed in *Achromobacter* grown in the absence of biotin (Höpner and Knappe, 1965), in which case 20% of the MCCase was in the apoenzyme form.

To test this hypothesis we determined the biotin content in leaves and roots of tomato plants. Results of the biotin assays with extracts from roots and leaves are summarized in Table 2. Total biotin content was higher in leaves than in roots, by a factor of 4. The majority of the biotin in both organs was free, not protein-bound. Protein-bound biotin accounted for 15-25% of the total biotin in both organs. Therefore, the

presence of apo-MCCase in tomato leaves was not due to the lower availability of biotin in this organ, but must be due to another mechanism.

The total biotin concentration in leaves of tomato is in the range of the reported concentration of biotin in siliques and cauline leaves of *Arabidopsis thaliana* (Shellhammer and Meinke; 1990). The biotin concentration in the rosette leaves of *A. thaliana* were slightly lower than our findings (about 20 ng/g for *A. thaliana*). As with our findings, Baldet et al., (1993), reported that the majority of the biotin in pea leaves and mesophyll protoplasts is free, not protein-bound. Interestingly, when we express our biotin concentration data on a per mg protein basis we obtain values that are approximately 10-times higher than those reported from pea (Baldet et al., 1993). The difference maybe due to the fact that these workers determined biotin content in isolated mesophyll protoplasts, whereas our determinations were done on the extracts from intact organs. The differences may imply that the majority of the biotin in plants may be extra-cellular.

Summary and Physiological Significance— The metabolic function of MCCase in plants is as yet to be established. Extrapolation from animals and bacteria would suggest that it is involved in the catabolism of leucine, and in the "mevalonate shunt" by which mevalonate can be metabolized to nonisoprenoid compounds. Radiotracer studies with plants provided with either ^{14}C -leucine (Stewart and Beevers, 1967) or ^{14}C -mevalonate (Nes and Bach, 1985) have provided evidence that leucine catabolism and the mevalonate

shunt operate *in planta*. However, the regulation of these two interconnected pathways and the significance of these pathways to the physiological status of the plant is unclear. Furthermore, the extent to which leucine maybe catabolized by an alternate peroxisomal pathway, which does not require MCCase (Gerbling and Gerhardt, 1989), is unknown.

The studies described herein were undertaken to begin to address some of these questions. We found the MCCase activity is highest in the roots, a nonphotosynthetic organ. In fact a cursory examination of the organ distribution of MCCase in tomato indicates an inverse relationship between photosynthetic competence of the organ and MCCase activity. This would be consistent with the idea that MCCase functions in leucine catabolism *in planta*, which may be higher in nonphotosynthetic organs to generate ATP and reducing equivalents by respiration of carbon derived from leucine. The catabolism of leucine for respiration in nonphotosynthetic tissues is probably part of a general process of catabolism for the purposes of respiration.

In attempting to uncover the mechanisms that generate a ten-fold difference in MCCase activity between leaves and roots, we discovered that differential biotinylation of the apoenzyme plays a significant role in regulating MCCase activity. The presence of apo-biotin enzymes have previously been reported in bacteria and animals (Kosow and Lane, 1961a; 1961b; 1962a; 1962b; Kosow et al., 1962; Jacobs and Majerus, 1970; Deodhar and Mistry, 1969a; 1969b). In these reports, the occurrence of apo-biotin enzymes was due to biotin deficiency. Thus

biotinylation was not a regulatory mechanism for the regulation of enzyme activity. Here, we report for the first time, that biotinylation is an important regulatory mechanism by which MCCase activity is differentially regulated in different organs of tomato.

The biochemical mechanism that leads to the differential biotinylation of MCCase in roots and leaves requires additional research. One potential mechanism for this maybe the expression of different isoenzymes of MCCase in leaves and roots, which differ in their ability to act as substrates for the holocarboxylase synthetase, that is the leaf MCCase isoenzymes maybe a poorer substrate for the holocarboxylase synthetase, than are the root MCCase isoenzymes. The presence of isoenzymes of MCCase has been reported in soybean (Song et al., submitted), however, there was no indication of differential biotinylation in that system. The other potential explanation for the different biotinylation state of MCCase, is that it is a product of differential activity by the holocarboxylase synthetase in roots and leaves.

The presence of a pool of apo-MCCase in leaves of tomato leads to a number of questions as to the nature of the apoenzyme. MCCase is present in mitochondria of animals (Hector et al., 1980) and plants (Baldet et al., 1992; Crane et al., in preparation); therefore, it will be interesting to ascertain where the apo-MCCase subunit accumulates in the cell. If it is located in mitochondria, is it incorporated into the holoenzyme, in which case what effect does it have on the enzyme? Research to address these questions will undoubtedly lead to new and

interesting information about the regulation and structure of biotin enzymes.

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Table I. Organ Distribution of MCCase in Tomato

Organs	MCCase Activity ^a	
	nmol/min mg protein	nmol/min g fresh weight
Leaves	4.9 ± 0.5	2.02 ± 0.23
Stems	5.8 ± 0.5	1.09 ± 0.33
Roots	40.3 ± 5.1	1.01 ± 0.30
Ripening Fruits	10.4 ± 1.2	0.92 ± 0.15
Ripe Fruits	24.5 ± 3.5	1.16 ± 0.19

^a Average of five determination

Table II. Biotin Content in Tomato Leaves and Roots ^a

Biotin	Leaves		Roots	
	<i>ng/g fresh weight</i>	<i>ng/mg protein</i>	<i>ng/g fresh weight</i>	<i>ng/mg protein</i>
Total	189 ± 20	18.0 ± 2.0	44 ± 7	15.6 ± 2.4
Bound	51 ± 6	4.9 ± 0.5	6.3 ± 1.1	2.2 ± 0.3
Free	130 ± 10	15.6 ± 2.8	28 ± 5	9.8 ± 3.3

^a Average of five determination

FIG. 1. Differential Accumulation of the Biotin Subunit of MCCase and its mRNA in Leaves and Roots of Tomato. Equal amounts of RNA (25 μ g) isolated from leaves (Lane 1) and roots (Lane 2) of tomato were fractionated by electrophoresis in a formaldehyde-containing agarose gel. The resulting gel was stained by EtBr (Panel A) or subjected Northern blot analysis and probed with a cDNA coding for the biotin subunit of tomato MCCase (BTN cDNA, Wang et al., submitted), (Panel B). Equal amounts of protein (20 μ g) extracted from leaves (Lane 1) and roots (Lane 2) of tomato were analyzed by SDS-PAGE. The resulting gels were subsequently subjected Western blot analyses. The filter was sequentially incubated with antiserum to the biotin subunit of tomato MCCase and 125 I-Protein A (see Material and Methods), (Panel C). After removal of the antibodies and 125 I-Protein A with 0.2 M Glycine-HCl (pH 2.2), 0.5 M NaCl, the filter was incubated with 125 I-streptavidin to detect biotin prosthetic group on the biotin subunit of MCCase (Panel D). Aliquots of extracts containing equal amount of MCCase activity isolated from leaves (Lane 1) and roots (Lane 2) of tomato were subjected to SDS-PAGE and subsequently Western blot analysis with 125 I-streptavidin (Panel E).

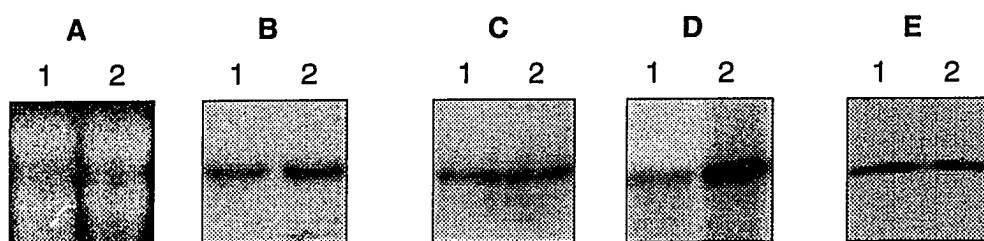
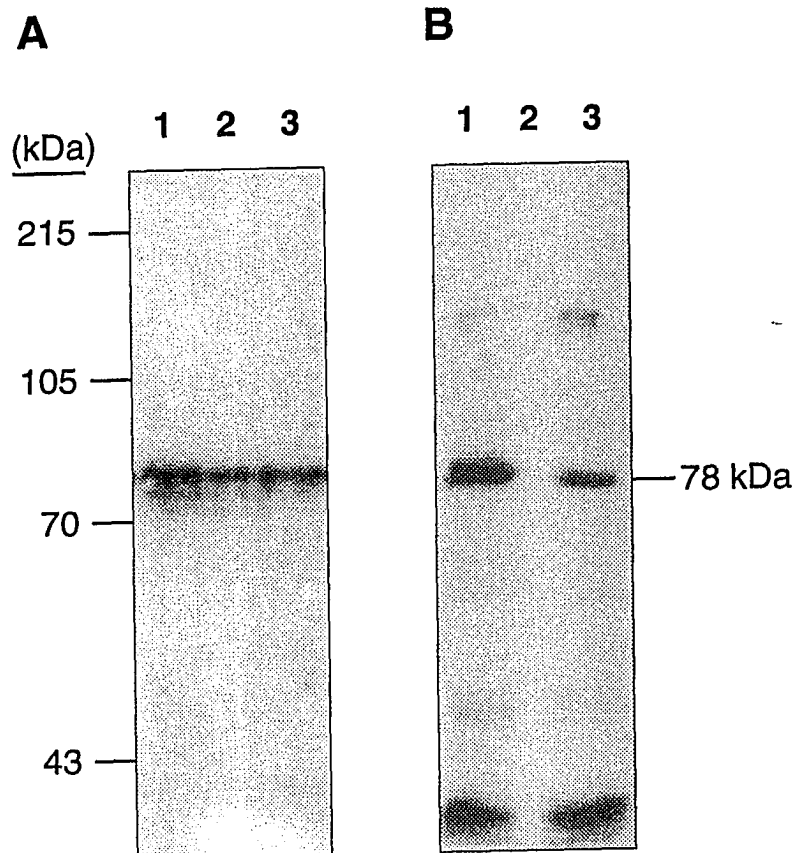


FIG. 2. Detection of Apo-MCCase in Leaves of Tomato. A denatured protein extract (Lane 1) from tomato leaves was subjected to affinity chromatography with immobilized avidin. Proteins that did not bind to the column were collected (Lane 2) and after extensive washing of the column biotin-containing polypeptides were eluted (Lane 3). These three fractions were subjected to SDS-PAGE and Western blot analysis. *Panel A*, the blot was sequentially incubated with antiserum to the biotin subunit of tomato MCCase and ^{125}I -Protein A. *Panel B*, the blot was incubated with ^{125}I -streptavidin to detect biotin-containing polypeptides.



GENERAL SUMMARY AND CONCLUSIONS

The genes and cDNAs coding for MCCase were isolated, for the first time, as described in this dissertation. This is also the first molecular cloning of genes and cDNAs coding for plant biotin enzymes. The original identification of the cDNA coding for the biotin subunit of MCCase was accidentally made by the fact that when the cDNA was expressed in *E. coli* its protein product was biotinylated (Hoffman et al., 1987). Sequencing of this cDNA indicated that it codes for a polypeptide which had Ala-Met-Lys-Met, a tetrapeptide that occurs in most of the biotin enzymes. These findings suggested that this cDNA might code for a tomato biotin enzyme. This hypothesis was particularly interesting since knowledge of biotin enzymes in plants was very limited and none of the genes or cDNAs coding for plant biotin enzymes were cloned.

To ascertain whether this cDNA was from a gene that codes for a biotin enzyme in tomato and to identify this enzyme, three longer cDNAs were isolated. The nucleotide sequences were determined from these cDNAs. They were also expressed in *E. coli* as chimeric proteins and these proteins were purified to homogeneity. The antisera generated against the expressed proteins from these cDNAs reacted solely with a 78 kDa polypeptide in tomato leaf protein extracts. This polypeptide was identified as a biotin-containing protein by the fact it specifically bound to avidin but not to the avidin which was preincubated with free biotin.

The enzymatic identity of this biotin-containing polypeptide was identified as MCCase. Aliquots of tomato leaf protein extracts containing ACCase, PCCase, MCCase and PCase activities were incubated with the antisera or preimmune serum to the expressed proteins from these tomato cDNAs. In these experiments, only MCCase activity was inhibited by the antisera, which demonstrates that the 78 kDa biotin-containing polypeptide is a component of MCCase. The conclusion was further supported by the purification of MCCase from tomato leaves.

Comparison of the deduced amino acid sequence from the cDNA clones of the biotin subunit of MCCase with other biotin enzymes suggests that the 78 kDa subunit of MCCase carries biotin carboxylase and biotin carboxyl carrier functions. The amino acid sequence in between of the two domains have low homology among biotin enzymes. Immunological studies indicate that this region does not participate catalytic process of the enzymes.

In addition, the predicted secondary structures of the amino acid sequence surrounding the biotinylation site were found to be highly conserved. Along with conserved secondary structures, a set of hydrophobic amino acids were found to be conserved among biotin enzymes. A hypothesis was proposed based on these findings that they might be the structural features that define the lysine residue for biotinylation by the holocarboxylase synthetase.

Two distinct genomic clones coding for the biotin subunit of MCCase were isolated from a tomato genomic library. Both genomic clones

were characterized in terms of restriction map and location of the sequences coding for the biotin subunit of MCCase. The biotin subunit is coded by a small gene family in the tomato genome, with at least two copies of the gene.

The tomato MCCase was partially purified from leaves. The enzyme is composed of two different types of subunits. The larger subunit contains biotin and has a molecular weight of 78,000. The smaller subunit does not contain biotin and has a molecular weight of 60,000. The molecular weight of the native MCCase was determined, by gel filtration chromatography and nondenaturing polyacrylamide gel electrophoresis to be in the range of 1,000 kDa -1,400 kDa.

In a survey of tomato organs, MCCase was found in all the organs examined. Its specific activity is highest in roots, followed in descending order by ripe fruit, ripening fruit, stems, and leaves. The apparent inverse relationship between MCCase activity in the organ and the photosynthetic capacity of that organ is consistent with MCCase having a role in leucine catabolism to generate ATP and reducing equivalents when these cannot be produced by photosynthesis directly.

The biochemical mechanism by which the 10-fold difference in MCCase activity between leaves and roots was investigated. These studies indicated that MCCase activity is regulated by the differential biotinylation of the enzyme, with biotinylation occurring more efficiently in roots than in leaves. This poor biotinylation of the apo-MCCase in leaves is not due to the lower concentration of biotin in this organ. Suggesting that biotinylation of apo-MCCase is a specifically

regulated process, probably via the regulation of hoiooxylase synthetase.

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